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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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THE FATE OF HUMAN AND BOVINE TUBERCLE BACILLI IN VARIOUS ORGANS OF THE RABBIT.

By MAX B. LURIE, M.D.

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(Received for publication, April 17, 1928.)

In a recent study of the susceptibility of various organs of the rabbit to tuberculosis (1) attention was drawn to the fact that their "native" cellular reaction to the intravenous injection of heat-killed tubercle bacilli differed greatly. From this and from other data the inference was drawn that different organs in a given species possess an inherently different power to destroy tubercle bacilli before any allergic manifestations are apparent. It was thought desirable to examine this problem more thoroughly.

Now, while dead bacilli elicit the cellular reaction to the chemical constituents of the organism, they do not reproduce all the conditions of infection with living bacilli. Dr. Eugene L. Opie suggested that the problem be attacked with living bacilli by cultural methods. Obviously this approach complicates the problem, but the results so obtained are more significant.

Cultural methods have often been used for the study of infectious diseases and for the isolation of tubercle bacilli from various sources, but the only instance of their use we know of, for the quantitative estimation of tubercle bacilli in a given source, is Lange's attempt (2) to estimate the number of living bacilli by the number of colonies that developed from a given suspension of tubercle bacilli planted on serum egg medium. After a few trials with (3) Boissevain's plasma method, which was found to be unsuitable for our purpose, preliminary experiments were performed with Dorset's egg medium and Petroff's gentian violet medium. It soon appeared that, while the method does not give the absolute number of living tubercle bacilli in a given specimen of tissue, it gives the relative numbers with sufficient accuracy. During the progress of this work Madsen and Mørch (4) have reported

a close correspondence between Petroff's method and guinea pig inoculation in their studies on rabbits treated with sanocrysin.

Method.

At varying intervals of time after the intravenous injection of varying quantities of different types of tubercle bacilli, the rabbit is killed by a blow on the head. The hair on the entire ventral surface of the animal is removed by a depilatory mixture and the skin washed and covered with an antiseptic. With rigorous precautions for sterility, the skin is reflected. The abdominal cavity is opened, a separate pair of sterile instruments being used for each step. The first organ that presents itself in the wound is the liver. 1 gm. quantity is weighed accurately into a sterile Petri dish. In a similar manner like weighed amounts of spleen and kidney are excised as they are successively uncovered. The thoracic cavity is now opened and 1 gm. quantity of lung, representative in its gross pathological condition of the entire organ, is removed. Aseptically, the right femur is crushed with forceps and a weighed amount, as near a gm. as possible, of bone marrow is removed. The specimens of tissue are then ground in sterilized mortars with sterilized sand as finely as possible consistent with sterility and are suspended in a given quantity of fifteenth molar disodium phosphate (5). From these suspensions, containing a known weight of a given tissue, dilutions are made in the same alkaline medium. 1 cc. quantity of each dilution of each organ is shaken in a sterilized tube containing glass beads and seeded directly on three tubes of Dorset's medium and three tubes of Petroff's medium, divided as equally as possible and spread as evenly as possible on the surface of the medium by means of a pipette. The suspended material is allowed to settle for at least ½ hour, often much longer. Uniform distribution of the inoculated material is secured by allowing the tubes to recline so that the surface of the medium is in a horizontal plane. The tubes are now incubated. Another 1 cc. specimen is treated with 3 per cent sodium hydroxide according to Petroff's method, centrifugalized, neutralized with hydrochloric acid, and seeded on three tubes of Petroff's and Dorset's medium respectively as before. After the tubes have been left in the incubator for varying periods of time, the number of tubercle bacillus colonies in each is counted and recorded at weekly or biweekly intervals. Repeated readings are necessary both to check the counts and also to prevent loss of any data that might result from subsequent contamination.

We found that at least 3 months must elapse before the ultimate number of colonies in any one tube could be determined, for often new colonies formed even as late as 10 weeks after incubation, especially with the bovine strain. Since the tubes must be maintained at incubator temperature for such a long time all precautions against drying out of the medium are necessary. Precautions must also be taken against the spreading of colonies by the liquid of condensation. For these reasons, the tubes were always read in an upright position and handled as

little as possible. When the number of colonies appearing on the surface of a tube was not more than 200 they could easily be counted directly. When the colonies exceeded this number, they were estimated by wrapping about the tube a piece of x-ray celluloid from which square windows of varying sizes had been cut out and reading these areas with a magnifying glass. Repeated readings tended to eliminate gross errors. In case of any doubt whether a given colony was of tubercle bacilli or not, smears were made and stained by the Ziehl-Neelsen method.

Strains.—Two human strains and one bovine strain were used. The human strains, P-15 B and P-48 A, were isolated in the latter part of 1923 from a nodule in the lung and from a hilum lymph node respectively of human autopsy material (6). Both were typical human strains as determined by rabbit inoculation. The bovine strain, Bovine C, was also isolated in 1923 by Dr. Joseph D. Aronson from a lymph node of a tuberculous cow. Originally it was exceptionally virulent for the rabbit (7) and it still behaves like a typical virulent bovine strain.

Dosage.—In one series of experiments the fate of the human tubercle bacillus, P-15 B, was compared with the fate of the bovine type, Bovine C, in the various organs of the rabbit in quantities of 0.1 mg. each to rabbits of approximately the same weight. In another series the behavior of the same bovine strain was compared with another human strain, P-48 A, in quantities of 0.001 mg. per kilo of body weight.

The animals were killed 2 days, 1, 2 and 4 weeks and 2 months after intravenous infection and at other intervals, as will be apparent from the tables and curves.

The figures in the tables represent the average number of colonies recovered, usually from three separate tubes, each planted with a like quantity of the same suspension of tissue of a given dilution. When two dilutions were used the figures represent the average of both. Since it required an entire day to perform a single experiment, for frequently as many as 120 tubes had to be seeded from a single animal, the same suspension of tubercle bacilli could not be used to inoculate all the rabbits of a given series. A given suspension was used to inoculate a number of rabbits corresponding to the number of intervals required, and similarly other suspensions, until the number of rabbits required for each interval was obtained. The slight variation in dosage thus introduced was lessened by preparing the suspensions as uniformly as possible from similarly aged cultures, etc. The same alkaline medium was used for making the suspension of tubercle bacilli as in suspending the tissues.

The Fate of Human Tubercle Bacilli in Large Dosage in the Various Organs of the Rabbit.

The number of colonies recovered from similar weights of lung, liver, spleen, kidney and bone marrow, 2 days, 1, 2 and 4 weeks and 2 months after injection of 0.1 mg. P-15 B into rabbits, after direct seeding upon Dorset's and Petroff's media and also after sodium hydroxide treatment following 3 months incubation, is recorded in detail in Table I.

TABLE I.

Number of Colonies Obtained from Organs of Rabbits Infected with 0.1 Mg. B. tuberculosis, Human.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.†	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
9-75	2 days	—	—	—	—	—	23	0	0	78		10	15	—	0	—	0	5	2	—	—
9-76	2 days	—	?	17	—	42	22	—	6	200	300	—	34	—	2	0	0	6	11	0	3
9-82	2 days	—	?	—	—	28	60	1	0	23	25	0	0	0	0	0	0	26	2	0	1
Average	2 days	—	?	17	—	35	35	1	2	100	162	5	16	0	1	0	0	12	5	0	2
9-77	1 wk.	—	—	32	35	225	—	9	0	—	—	13	4	2	4	0	—	94	0	1	0
9-84	1 wk.	—	—	—	—	225	80	—	—	825	585	—	—	—	—	—	—	13	3	—	
9-88	1 wk.	—	—	125	22	400	150	10	4	∞	875	62	37	31	5	0	0	64	58	1	0
Average	1 wk.	—	—	78	28	283	115	9	2	1,000	730	37	20	16	4	0	0	57	20	1	0
9-85	2 wks.	—	—	275	237	600	—	32	31	∞	∞	416	600	87	172	0	0	100	96	1	0
9-90	2 wks.	∞	∞	18	20	100	—	1	1	∞	∞	2	1	—	—	0	—	—	—	7	4
9-78	2 wks.	∞	∞	275	50	—	—	22	13	∞	1,185	5	0	—	37	9	0	333	75	33	4
Average	2 wks.	∞	∞	189	102	350	—	18	15	∞	∞	141	200	87	104	3	0	216	85	14	3
9-79	4 wks.	∞	∞	420	250	640	11	2	2	700	258	1	16	85	37	13	22	277	88	15	13
9-86	4 wks.	∞	∞	1,183	1,500	27	0	1	1	366	—	1	7	630	500	0	16	33	18	12	0
R 9-92	4 wks.	∞	1,200	—	—	—	—	0	0	300	20	—	1	545	283	—	4	57	16	1	0
Average	4 wks.	∞	∞	801	875	333	5	1	1	455	139	1	8	420	273	6	14	122	40	9	4
9-87‡	2 mos.	13,750	10,000	555	—	1	0	0	—	2	0	0	—	816	1,066	21	—	213	18	0	—
10-12‡	67 days	66	—	16	45	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
15-01‡	60 days	50	18	2	1	12	0.5	6	3	22	—	20	6	68	38	41	3	5	1	1	0
Average	2 mos.	4,622	5,009	191	23	4	0	2	1.5	8	0	6	3	294	368	20	1.5	72	6	0.3	0

* D. = Dorset's medium.

† P. = Petroff's medium.

‡ Computed from dilutions 1:10 and 1:100 instead of 1:6 as in all remaining figures in this table.

Mathematical accuracy is not to be expected in a biological experiment of this nature, especially in dealing with the capricious growth proclivities of an organism like the tubercle bacillus. Furthermore, variations in the susceptibility of individual rabbits, in the pieces of tissue selected, and in the dispersion of the tubercle bacilli in the inoculum, caused variations in the results obtained. The method is nevertheless adequate for the problem on hand.

The arithmetical averages of the number of bacilli recovered from each organ of three separate rabbits are plotted in the accompanying curves (Charts I and III).

Original Deposition.—2 days after the intravenous injection of bacilli, the number of colonies recovered from like quantities of the various organs of the rabbit is similar to the relative distribution of India ink after intravenous injection (8).

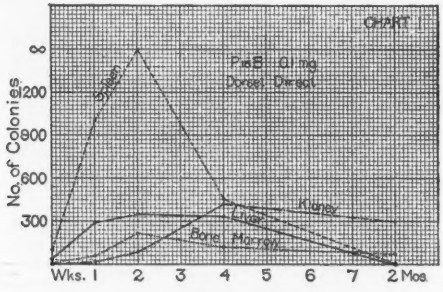

CHART I.

Thus from the spleen, an average of 100 colonies was recovered from 25 mg. of tissue on Dorset's medium, then 35 colonies from the liver, next 12 from the bone marrow, and none from the kidney. Essentially parallel results were obtained from Petroff's medium after direct seeding and from both media after sodium hydroxide treatment. Of course smaller numbers of colonies were recovered after treatment, but since we are not concerned with absolute but with relative quantities each of these data serves as a check for the other.

It is difficult to isolate tubercle bacilli in pure culture from the lung without sodium hydroxide, until pathological changes have taken place. For this reason it is not certain from the data in the table what is the relation of the lungs to the other organs as regards the deposition of tubercle bacilli. Apparently it is other than can be accounted for by the distribution of particulate matter.

1 week after the infection there is a rapid multiplication of the bacilli in the different organs, but not to the same degree in all the organs. The spleen shows

the most rapid multiplication; the liver much less. An increased number of bacilli is also discernible in the bone marrow and kidney.

This increase in all the organs continues unabated in the *2nd week* after injection.

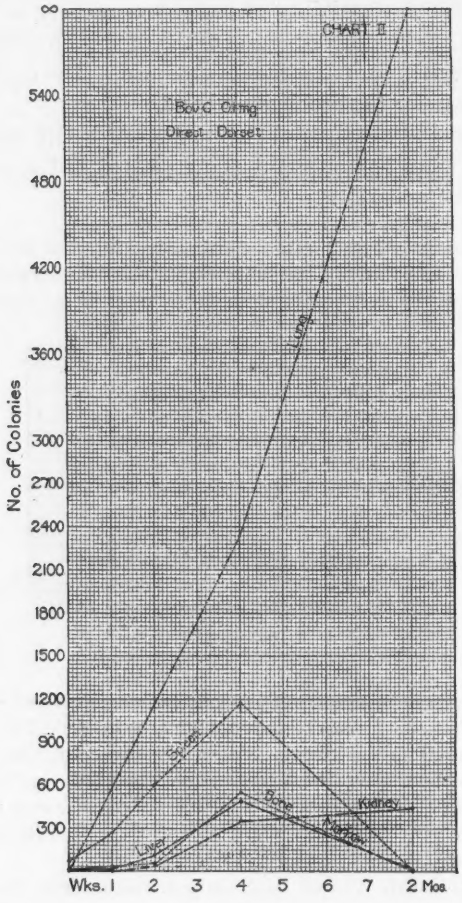

CHART II.

At this time, the relative numbers of colonies to be recovered from a given organ bear only a superficial relationship to the original deposition in that organ (see Table I).

4 weeks after infection, a striking change takes place. The picture differs greatly in the spleen, liver and bone marrow on the one hand, and in the lung and kidney on the other. In the first group of organs, especially in the spleen, there is not only a cessation of growth but a marked destruction of tubercle bacilli, as is indicated by the far fewer numbers of colonies recoverable from them after 4 weeks than after 2 weeks. There are still, however, large numbers of tubercle bacilli remaining in these organs. In the lung and kidney, on the contrary, the increase in the number of bacilli continues unabated.

2 months after infection the destruction is almost complete in the liver and spleen and comparatively few bacilli can be isolated from the bone marrow. But it is apparent that the rate of destruction between 4 and 8 weeks is much slower than

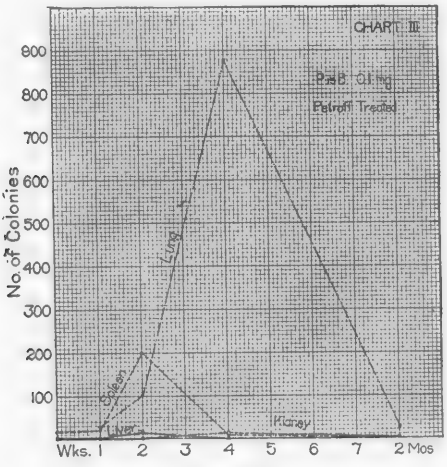

CHART III.

between 2 and 4 weeks. Destruction also becomes apparent in the lung, and cessation of growth, if not destruction, in the kidney.

The factor of individual resistance of the rabbit to tuberculous infection is especially noticeable in the 2 month interval. Compare the number of colonies isolated from the several organs of Rabbit 98–7 on the one hand and Rabbits 10–12 and 15-01 on the other. Such variations sometimes make the interpretation of data difficult.

It is seen then, that during the first 2 weeks after infection with 0.1 mg. of P-15 B the tubercle bacilli multiply in all the organs. Although their rate of increase differs in the different organs, there is as

TABLE II.

Number of Colonies Obtained from Organs of Rabbits Infected with 0.1 Mg. B. tuberculosis, Bovine.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.†	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
9-98	2 days	—	—	0	0	10	15	0	0	66	7	—	0	0	0	0	0	0	0	0	0
17-62‡	2 days	5	0	0	0.8	10	0	4	4	70	0	0	2	—	—	0.5	0	20	0	0	0.5
Average	2 days	5	0	0	0.4	10	7	2	2	68	3.5	0	1.0	0	0	0.2	0	10	0	0	0.2
9-94	1 wk.	—	0	—	0	—	0	0	0	57	4	0	1	0	0	0	0	18	2	1	0
9-99	1 wk.	—	0	—	0	20	0	1	0	261	0	0	0	1	0	0	0	78	0	1	0
10-00	6 days	—	—	0	0	3	1	0	0	501	5	0	0	1	1	0	0	1	0	0	0
Average	1 wk.	—	0	0	0	12	1	1	0	273	3	0	1	1	1	0	0	32	1	1	0
9-95	2 wks.	—	0	2	2	118	0	1	3	416	1	0	0	—	—	0	0	49	1	0	1
10-01	2 wks.	—	—	12	45	61	0	1	10	800	0	1	3	22	44	3	0	28	17	0	1
10-05	2 wks.	—	—	0	0	180	0	1	0	600	0	0	0	57	0	0	0	88	0	1	0
Average	2 wks.	—	—	5	16	119	0	1	4	605	1	1	1	39	22	1	0	55	6	1	1
99-6	4 wks.	795	0	46	0	27	0	23	12	225	0	3	0	4	0	0	0	167	1	—	0
10-02‡	4 wks.	1,200	1,200	16	105	34	2	0	0	918	100	3	4	241	0	0	0	103	100	1	7
10-06‡	4 wks.	5,000	12,200	330	383	1,396	216	65	33	2,375	0	33	166	798	0	0	0	1,385	276	40	120
Average	4 wks.	2,331	4,466	130	162	485	72	29	15	1,172	33	13	57	347	0	0	0	551	125	20	42
9-97‡	2 mos.	1,666	1,120	233	85	27	0	4	3	16	0	0	0	21	0	4	1	0	0	0	0
11-59‡	64 days	∞	∞	∞	75,000	6	0	0	0.5	2	0	3	0	883	510	595	945	0.5	0	0	0
Average	2 mos.	∞	∞	∞	∞	16	0	2	1.7	9	0	1.5	0	452	255	299	473	0.2	0	0	0

* D. = Dorset's medium.

† P. = Petroff's medium.

‡ Computed from figures for 1:10 and 1:100 dilutions; all other figures represent dilution 1:6.

yet no effective opposition to the growth of the virus. Furthermore, the subsequent arrest in multiplication followed by destruction of the bacilli takes place about simultaneously in the three physiologically and anatomically very different organs, the spleen, liver and bone marrow. The only function these organs have in common is to remove particulate matter or bacteria from the blood stream, a func-

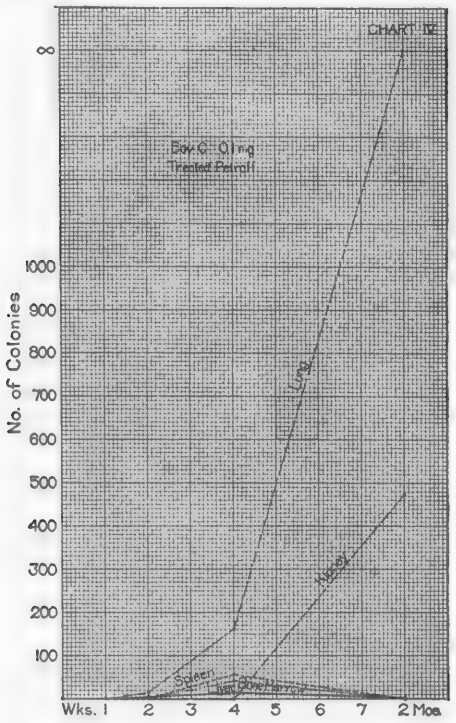

CHART IV.

tion shared to a much smaller degree by the lung and kidney, where destruction takes place later. Again this turn in the fate of tubercle bacilli does not depend upon the quantity of tubercle bacilli accumulated in a given organ. Thus in the spleen they reach uncountable numbers before destruction begins but only moderate numbers in the liver and bone marrow.

The majority of the animals recovered from the disease at the end of 2 months, though they were far from completely eliminating the bacilli.

The Fate of Bovine Bacilli in Large Dosage in the Various Organs of the Rabbit.

Table II and Charts II and IV show the fate of bovine tubercle bacilli in various organs of the rabbit at different intervals of time after the intravenous injection of 0.1 mg. of Bovine C, which is comparable in all respects to the fate of P-15 B.

One striking difference is apparent at once: *2 days* after bovine infection far fewer colonies were isolated from like quantities of tissue. Yet the relative distribution in the various organs is again found to be similar to that of particulate matter; namely, spleen, liver, bone marrow and kidney. In Rabbit 17–62 the deposition in the lung was less than in the bone marrow.

It was found that the growth *in vitro* of this virulent bovine organism is much more subject to unfavorable influences than the human type P-15 B. Yet when these two strains are transplanted on glycerol agar in the usual way, they grow equally fast and luxuriantly. It is possible that this difference in behavior may be attributed not to different *in vivo* relations but simply to the original dysgonicity of the bovine bacillus, which comes into play when the bacilli are sufficiently dispersed. It may be for this reason that fewer colonies are isolated from tissues infected with Bovine C, and not because fewer viable bacilli are present. This problem is being investigated separately. However, as can be seen from the tables, very large numbers of colonies can be isolated even from Bovine C at times.

1 week after infection there is an increase in the number of colonies to be recovered from the various organs, but the relative rise is much less than with P-15B.

Without attaching too much significance to the actual numbers, it is permissible to draw attention here to the following relationships. 1 week after infection, 5 days after the first interval, the human strain, seeded directly on Dorset's medium, showed an increase of about eight times in the liver, ten times in the spleen, five times in the bone marrow, and sixteen times in the kidney, while the bovine strain similarly cultured showed hardly any increase in the liver and kidney, and an increase of three times in the bone marrow and four times in the spleen.

Now even if we admit that the bovine strain is dysgonic on reisolation from the tissues so that we start with a lower base line for this strain, still if its rate of growth in the body were the same as the human strain's, the relative increase would be the same in the various organs. Therefore we are forced to the conclusion that during the 1st week the bovine strain grows more slowly in the body of the rabbit than the human strain. With both strains the rate of multiplication of the bacilli in the spleen is much faster than in the liver.

2 weeks after infection the increase continues in all the organs, just as with the human strain.

The bovine bacilli reach their highest numbers in the liver, spleen and bone marrow *4 weeks* after infection, at a time when the human bacilli show a decided decrease.

A large percentage of the rabbits thus infected died before the close of the next interval. Out of seven rabbits, only two survived 2 months. The remaining five died on an average of 38 days after infection. Two died on the 24th and 25th day after infection, at a time when the multiplication of the bacilli in all of the organs was going on unchecked. Thus it is seen that, with large doses of bovine tubercle bacilli, death may take place before any effective opposition to the growth of the parasite in any of the organs is realized. Individual rabbits of greater resistance may survive long enough for the altered reaction of the tissues toward the virus to become apparent.

2 months after infection we find the disappearance, almost but not quite complete, of the tubercle bacilli in the liver, spleen and bone marrow, the organs that showed the earliest change in behavior toward the human infection. At the same time the animal is dying from pulmonary and renal infection. For in contradistinction to the fate of the human strain, the lung and kidney still show an unhindered multiplication, especially the lung, where the tissue becomes veritably a pure culture of tubercle bacilli. Thus even the resistant animal succumbs.

The chief difference between the human and bovine types, therefore, is the slower multiplication of the bovine bacilli and the later appearance of their destruction. Although the difference is quantitative rather than qualitative it is sufficient to cause death after bovine infection, because of the continued multiplication in the lung and kidney, and regression of the lesion and survival after human infection.

The Fate of Human Bacilli in Small Dosage in the Various Organs of the Rabbit.

The human tubercle bacillus, then, grows more rapidly in the body of the rabbit than the bovine, and this more rapid growth is followed

TABLE III.

Number of Colonies Obtained from Organs of Rabbits Infected with 0.001 Mg. B. tuberculosis, Human, per Kilo.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.†	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
11-40	30 min.	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
11-61	30 min.	30	0	5	5	3	1	1	0	6	3	1	1	0	0	0.15	0	0	0	0	0
11-71	3 hrs.	3	2	0	1	0	0	0	1	1	0	3	2	0	0	0	0	0	0	0	0
11-62	1 day	—	—	1	1	2	0	1	2	4	1	1	1	0	0	0	0	0	0	0	0
12-25	1 day	9	11	5	2	1	0	2	3	9	10	7	10	0.15	0	0	0	0.15	0.3	0.3	0.15
11-46	2 days	—	3	0	0.5	1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
12-28	2 days	15	9	3	6	—	0	2	5	8	3	3	3	0	0.15	0	0	0.15	0	0	0
Average	Short intervals	11.4	4.1	2.0	2.2	1.5	0.1	0.8	1.5	4.5	2.4	2.1	2.4	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0
11-42	1 wk.	3	3	0	0	—	0	0.3	0	8	0	0	0	0	0	0	0	2	0	0	0
11-47	1 wk.	6	6	3	0	3	0	0.3	0.3	6	6	0.6	1.6	0	0	0	0	0	0	0	0
12-26	1 wk.	43	105	9.5	3	3	0.3	0	0	29	10	4.3	2.6	0	0	0	0	8	1.3	0.3	0.6
Average	1 wk.	17	38	4.1	1	3	0.1	0.2	0.1	14.3	5.3	1.6	1.4	0	0	0	0	3.3	0.4	0.1	0.2
11-48	2 wks.	63	50	33	15	12	0	2.6	1.3	211	30	11	18	0	0	0	0	0	0.3	0	0
11-74	2 wks.	—	—	46	47	30	34	14.8	8.3	844	390	150	143	0	1.8	0	1	17	16	1.3	4.5
10-86	2 wks.	870	330	78	129	88	86	9	16	1,850	1,630	244	180	1.3	0	0.3	0.3	25	32	14	11
Average	2 wks.	466	190	52	63	43	40	8.8	8.5	968	683	135	113	0.4	0.6	0.1	0.4	14	16.1	5.1	5.1
11-73	4 wks.	10,660	8,000	5,400	4,530	139	120	22	36	6,330	4,160	870	1,236	1.6	0	0.3	1.0	220	270	43	38
11-75	4 wks.	415	416	236	202	1.7	0	1	2	498	280	158	142	0	0	0	0	13	11	8	13
11-51	4 wks.	—	—	2,560	3,100	3	0	2	0	204	163	44	16	5	6.6	9	2	9	5.5	2.5	1.6
Average	4 wks.	5,537	4,208	2,732	2,610	47	40	8	12	2,344	1,534	357	464	2	2	3	1	80	95	17	17

11-57	2 mos.	151	94	98	121	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
9-41	2 mos.	4,600	6,400	3,250	2,700	—	0	0	0	15	0	0	1.6	210	50	81	145	0	0	0	0
12-27	2 mos.	2,230	4,750	2,500	4,430	0	0	0	0.3	190	1.5	21	35	246	196	180	280	0	0	0	0
Average	2 mos.	2,327	3,748	1,949	2,417	0	0	0	0.1	69	0.5	7	12	152	82	87	141	0	0	0	0
13-09	4 mos.	—	—	—	0.5	—	—	0	0	—	—	0	0	—	0	0	0	0	0	0	0
13-19	4 mos.	—	785	690	390	—	—	—	0	0	0	—	—	—	—	0	0	0	0	0	0
14-48	4 mos.	—	20	17	0	0	—	0	0	—	—	0.3	2	—	0	0	0	—	—	0	0
Average	4 mos.	—	402	353	130	0	—	0	0	0	0	0.15	1	—	0	0	0	0	0	0	0
14-56	6 mos.	—	200	28	21	0	0	0	0	52	33	33	9	0	0	0	0	0	0	0	0
14-52	6 mos.	50	53	—	29	0	0	0	0	36	2	37	45	—	0	0	0	0	0	0	0
14-47	6 mos.	10	—	3	—	0	—	0	—	0	—	0	—	0	—	0	—	0	—	0	—
Average	6 mos.	30	126	15	25	0	0	0	0	29	17	23	27	0	0	0	0	0	0	0	0

* D. = Dorset's medium.

† P. = Petroff's medium.

by a more rapid destruction. The question arose whether the phase of destruction would be deferred if the accumulation of human tubercle bacilli were retarded by injecting smaller doses.

A series of rabbits was inoculated intravenously with 0.001 mg. per kilo of a human strain, P-48 A. This dose was chosen because it was thought that human

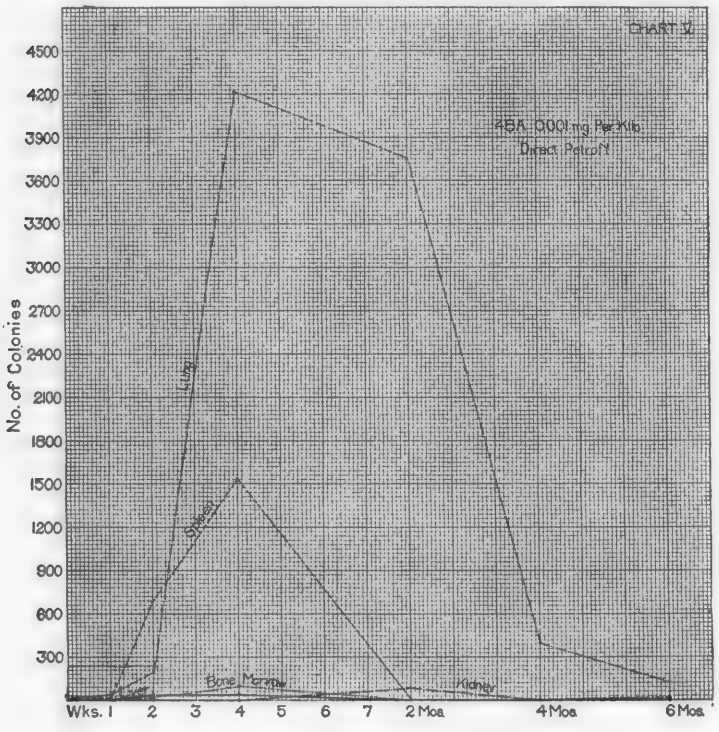

CHART V.

bacilli in a smaller quantity might be destroyed by virtue of the native reaction, and this strain, to see whether the relations would hold for a different strain.

The animals were killed at the following intervals: 30 minutes, 3 hours, 1 and 2 days, 1, 2 and 4 weeks, 2, 4 and 6 months. With the exception of the short periods at least three rabbits were used for each interval. The organs were cultured as above described. The detailed results are given in Table III and the averages are plotted in the curves of Chart V.

Original Distribution.—During the first *2 days* the greatest number of colonies was recovered from the lung, next from the spleen, next from the liver, and as a rule none from the bone marrow and kidney. No significant difference in distribution was noted if the rabbits were killed 30 minutes, 3 hours, 1 day or 2 days after infection. It is noteworthy that again, as apparently with the large doses of human bacilli, more colonies are recovered from the lung than from the spleen and liver, a distribution consistently at variance with the distribution of particulate matter. The relative distribution to the other organs follows that of manganese dioxide and carbon, namely, spleen, liver, bone marrow and kidney. Since even 30 minutes after injection the bacilli were recovered in greater numbers from the lung than from the other organs, the phenomenon cannot be due to a rapid and immediate multiplication in the lung and a slower initial multiplication or more rapid destruction in the other organs, but must be due to a greater deposition in the lung. No explanation can well be offered with the data at hand.

1 week after infection there is scarcely any rise in the number of bacilli recoverable from the various organs. This is in contrast to the sharp rise in the 1st week after the injection of 0.1 mg. P-15 B, and shows the same latent period noted after the injection of 0.1 mg. Bovine C.

2 weeks after infection there is a marked rise in the number of bacilli recoverable from the spleen and lungs. In the liver and bone marrow the increase is much less, and it is scarcely perceptible in the kidney. Here the striking difference in the behavior of tubercle bacilli in the several organs comes to the fore. The slight rise in the bone marrow and in the kidney can easily be attributed to the very slight original localization of bacilli in these organs. But the increase in the liver cannot, for here the original deposition was only 3 to 4 times less than in the spleen, and by the end of the 2nd week an average of 968 colonies was recovered from the spleens of three animals on Dorset's medium as against an average of only 43 colonies from their livers.

4 weeks. At this interval not only is there no destruction of the tubercle bacilli, such as was found in the spleen, bone marrow and liver 4 weeks after the introduction of 0.1 mg. P-15 B, but the bacilli are increasing in all the organs. However, the rate of increase in the interval from the 2nd to the 4th week is much slower than in the interval from the 1st to the 2nd week. It is noteworthy that the peak is reached in the spleen with over 2000 bacilli, but in the liver with only 47, showing again that the liver of the rabbit is far more unfavorable soil for tubercle bacilli than the spleen. Tremendous numbers of tubercle bacilli are recovered from the lung.

2 months after infection there is complete destruction of the bacilli in the liver and bone marrow, a marked reduction in the number recoverable from the spleen, and a considerable reduction in the lung. The drop in these organs appears to be simultaneous but probably only because of the length of time between intervals. It was unfortunately not forseen that observations 6 weeks after infection would have been desirable. In the kidney, the bacilli are still on the increase. It is

noteworthy that with the much smaller quantity of human bacilli, 0.001 mg., the destruction is not so complete at 2 months in the spleen as with the much larger dose, 0.1 mg. With the latter, an average of only eight colonies was recovered from the spleens of three rabbits at this time, but with the small dose an average of 69 colonies was recovered from somewhat smaller quantities, 16 mg., of the spleen on Dorset's medium. On the other hand the destruction is more complete in the bone marrow than with the larger dose.

Thus the chief differences appear in the behavior of a small quantity of human tubercle bacilli as compared with a large quantity of an organism of the same type though not the same strain. (1) There is a latent period of a week with the small dose; with the large dose the increase is immediate. (2) The destruction of tubercle bacilli begins later with the small dose than with the large dose. (3) At a given time after infection, more complete destruction is apparent after the large dose than after the small dose, at least in the spleen. Some organs, such as the liver and bone marrow, will show only slight numbers of bacilli even when their numbers are at their height after the small dose; after the larger dose the same organs will show considerable numbers.

Since none of the animals infected with the small dose of bacilli died from their tuberculous affection it seemed advisable to follow the fate of the bacilli in the various organs to their extinction, if possible.

4 months after infection the bacilli had been greatly reduced in the lung; they could no longer be isolated from the liver, kidney and bone marrow. Only a single stray colony could be recovered occasionally from the spleen, in which again, even 4 months after infection, the destruction of bacilli is not quite complete. Unfortunately these organs were cultured during the summer heat and many tubes appeared to be incompletely sterilized, although the methods used had heretofore been totally satisfactory in securing sterile media. However, sufficient data were obtained to assure us of the general trend of events.

6 months after infection the number of bacilli recoverable from the lungs is still further reduced. It is noteworthy that in the interval between the 2nd and 4th months the rate of destruction of the tubercle bacilli in the lung is very much faster than during the 2 months that follow. Thus on Petroff's medium after direct seeding the decrease in the 2nd to the 4th month was about 9 times, during the next 2 months a little more than 4 times. From the liver, kidney and bone marrow no tubercle bacilli were isolated 6 months after infection, just as none were recovered from these organs 4 months after infection. However, in the spleen a second slight increase became apparent.

Thus whatever force is responsible for the change bringing about the destruction of the tubercle bacillus weakens as time progresses. As a result the destruction is rapid at first, and becomes slower with time, and as early as 6 months after infection with 0.001 mg. P-48 A per kilo the bacilli have again begun to increase in the spleen while they are still decreasing in the lung. Possibly an adaptation of the parasite to the host has been effected.

The Fate of Bovine Tubercle Bacilli in Small Dosage in the Various Organs of the Rabbit.

A series of animals was infected intravenously with 0.001 mg. Bovine C per kilo. The detailed findings are presented in Table IV, and the averages are plotted in the accompanying curves, Chart VI.

With the smaller as with the larger dosages, fewer tubercle bacilli were isolated from the organs of rabbits infected with the bovine, than from those infected with the human strain. In harmony with this, and also with the relative distribution of particulate matter, the tubes seeded with suspensions of bone marrow and kidney 1 and 2 days after bovine infection with small doses, remained sterile in each case, and only occasionally and irregularly was a stray colony isolated from the lung and liver.

1 week after infection a slight rise in the number of colonies recovered was perceptible in the lung and spleen, while the liver, kidney and bone marrow gave practically all negative results in each of the three rabbits. This essentially parallels the latent period observed with a similar dose of a human strain.

2 weeks after infection there is a considerable rise in the number of colonies recoverable from the various organs. But the rise is much slower than with human bacilli in the same dosage. Thus between the 1st and 2nd weeks after infection with the human bacilli in small doses, the increase in the number of colonies recovered from the spleen was 69 times on Dorset's medium and 136 on Petroff's medium after direct seeding, but with the bovine, it was 8 times on Dorset's medium and 31 times on Petroff's. Like relations obtained in the other organs. It is noteworthy that very few bacilli were recovered from the liver of rabbits infected with small doses of either human or bovine strain; with the latter the number was negligible. On the other hand, the bacilli attain large numbers in the spleen of even bovine-infected animals and reach very large numbers in the spleens of rabbits infected with human bacilli. This discrepancy is altogether out of proportion to the relative original deposition in these two organs and seems to justify the conclusion that even in the "native" state the liver of the rabbit can check the growth of the bacilli much more completely than the spleen. At the end of the 2nd week some multiplication is also apparent in the lung and bone marrow. The kidney, however, still fails to show any bacilli.

TABLE IV.

Number of Colonies Obtained from Organs of Rabbits Infected with 0.001 Mg. B. tuberculosis, Bovine, per Kilo.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.†	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
11-83	1 day	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13-15	1 day	—	—	0	0	0	0	0	0	1.6	0	0	0	0	0	0	0	0	0	0	0
11-86	1 day	—	—	0.3	0.3	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0
11-85	2 days	—	—	0	0.3	0	0	0	0	0	0	0	0	—	0	—	0	0	0	0	0
13-35	2 days	—	—	0	0	0	0	0	0	1	0	0.3	0	—	—	0	0	0	0	0	0
12-30	2 days	1	0	0	0	0.3	0	0	0	7.5	0	0	0	—	0	0	0	0	0	0	0
Average	1 and 2 days	—	—	0	0.1	0	0	0	0	1.7	0	0.0	0	0	0	0	0	0	0	0	0
12-34	1 wk. (8 days)	—	—	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	0	0	0
13-29	1 wk.	—	0	0	0.3	0	0	0	0	4.3	0	0	0	0	0	0	0	0	0	0	0
12-32	1 wk.	47	0	7.6	20	0	0	0	0	20	0	2.3	0	—	—	0	—	1.6	0	0	—
Average	1 wk.	47	0	2.5	6.7	0	0	0	0	8.5	0	0.7	0	0	0	0	0	0.5	0	0	0
12-17	2 wks.	—	22	1	2	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
13-30	2 wks.	—	20	3	1.8	0	0	2	2.3	5.6	0	0	0	0	0	0	0	0	0	0	0
12-33	2 wks.	39	40	10	24	30	0	0	0	185	93	22	33	—	0	0	0	9.5	10	1.8	1.3
Average	2 wks.	39	27	4.6	9.2	10	0	0.6	0.7	64	31	7.3	11	0	0	0	0	3.1	3.3	0.6	0.4
11-89	4 wks.	60	85	8.5	2.5	0	0	0	0	30	16	35	0	0	0	0	0	0.5	0	0	0
13-36	4 wks.	30	—	13	29	0	0	0	0	705	625	40	107	44	0	9	6.5	21	6	0	7
12-36	4 wks.	257	750	52	91	3	0	0	3	750	916	305	385	105	0	8	0	5	0	2	3
Average	4 wks.	115	417	24	40	1	0	0	1	495	519	126	164	49	0	5.6	2.1	8.8	2	0.6	3.3

12-38	2 mos.	800	600	583	500	—	0	0	0	330	90	325	195‡	—	6	340	177	0	0	0	0
12-31	2 mos.	—	290	23	28	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12-37	2 mos.	70	—	4	13	0	0	0	0	—	—	0	0	—	—	0	0	—	—	0	0
15-26	2 mos.	1,155	4,465	197	770	0	0	0	0.6	1.5	—	0	0	105	90	3	3	0	0	1.5	0
Average	2 mos.	675	1,785	202	328	0	0	0	0.1	110	45	81	49	52	32	85	45	0	0	0.3	0
15-04	4 mos.	—	650	220	117	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
15-08	133 days‡	5,250	6,000	4,400	2,800	0	0	1	0	4	0	1	0.3	2,160	1,000‡	605	965	0	0	0	0
Average	4 mos.	5,250	3,325	2,310	1,458	0	0	0.5	0	2.5	0	0.5	0.1	1,080	500	302	482	0	0	0	0

* D. = Dorset's medium.

† P. = Petroff's medium.

‡ Probable figure.

4 weeks after infection, the bacilli have reached their highest numbers in the spleen and bone marrow. The growth in the lung continues unabated. For the first time since infection the bacilli appear in considerable numbers in the kidney. They were isolated from the liver of only one rabbit. In general the fate of the bacilli of the bovine and human strains is similar at this time, with, however, the following exceptions. With the human strain the number of bacilli in the lungs has gone far beyond those of the spleen. With the bovine strain the bacilli in

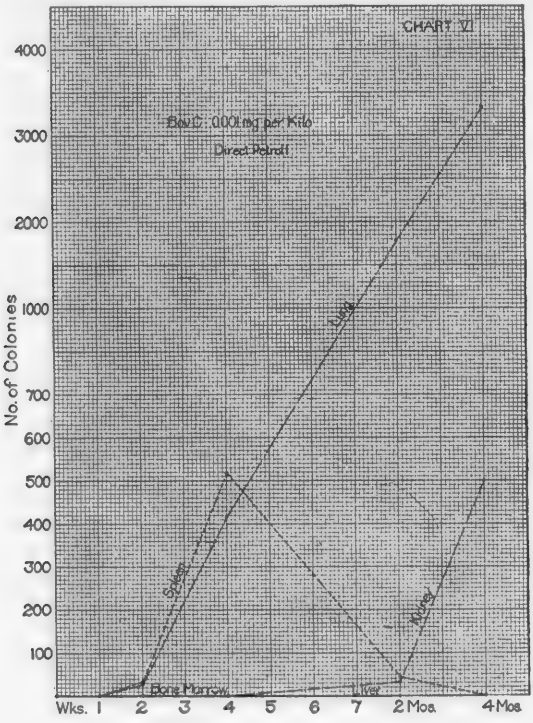

CHART VI.

the lungs still lag considerably behind those in the spleen, pointing again to the relatively slower multiplication of the bovine bacilli. Furthermore, the bacilli of the human strain reach higher numbers in the liver and bone marrow than do the bovine, so that the latter may be negligible while the former, even in the liver, attain considerable numbers. On the other hand, in the kidney the bovine bacilli have reached considerable numbers at this time, at least on Dorset's medium, whereas the human bacilli still are negligible.

2 months after infection the difference between the behavior of the human and bovine strains in this dosage is very striking. In the human strain the lungs have not only checked the further growth of the bacilli but they show considerable reduction in their numbers. The bovine strain, on the other hand, still continues to grow and multiply without any effective opposition. In both cases the spleen has effectively and markedly reduced the number of viable bacilli recoverable from similar quantities of tissue; however, the destruction is much more complete in the spleen of the rabbit infected with the human strain than with the bovine strain. With both types the bacilli have almost completely disappeared from the bone marrow and liver, in which the destruction is much more complete than in the spleen although these three organs generally parallel each other in behavior. In the kidney, both types of bacilli continue to increase.

4 months after infection the difference in the fate of the two types in small doses is most pronounced in two organs, the lung and kidney. While the bacilli of the P-48 A strain continue to be destroyed in the lung in this period the bovine bacilli are still increasing in numbers. Thus the drop in the lung begins in the 2nd month with the human infection and continues from then on, but the bovine bacilli continue to increase in number even 4 months after infection. Again the kidney after human infection shows complete disappearance of the bacilli by the 4th month, while bovine bacilli continue to multiply in this organ unabated. The destruction of bovine bacilli is also not quite so complete in the spleen as the destruction of human bacilli in the same interval. However, in both instances, the destruction is quite complete in the liver and bone marrow. The majority of the rabbits developed an extensive tuberculosis at the time of killing from which they would probably have died.

Thus we see, with the small doses of bovine as with the large doses, that one group of organs more or less completely destroys the bacilli—the liver, bone marrow and spleen—and the second group—the lung and kidney—is unable to check their growth. As between the bovine and human strains in small doses, we see the same difference that appeared between the human and bovine strains in large doses: slower growth and slower destruction of the bovine bacilli.

DISCUSSION AND SUMMARY.

In this study the attempt has been made to follow the fate of tubercle bacilli in the lung, liver, spleen, kidney and bone marrow of rabbits infected intravenously with large and small doses of human and bovine tubercle bacilli by determining the number of colonies recoverable from similar quantities of tissue on egg media at varying intervals during the course of infection. This method offers certain possibili-

ties for the elucidation of this problem precluded by the modes of attack used hitherto. Histological methods, while giving precise data in regard to tissue changes produced by the tubercle bacilli, are poor instruments for determining the fate of the bacilli in a given organ. Without stressing the notorious difficulties in staining the organism at all times, histological technique can give no definite answer to the question whether certain stained bacilli are living or dead, and it is the number of living bacilli that is of importance. Again animal inoculation, while an excellent index of the presence of living virulent bacilli, is a very inaccurate index of the number of living bacilli in a given specimen of tissue, for it is possible to infect guinea pigs with even a very few bacilli.

Lewis and Sanderson (9) have studied the histological changes in the rabbit's lung at varying intervals of time after the intravenous injection of similar quantities of human and bovine tubercle bacilli. They found that up to the 8th day tubercle bacilli are not abundant in either series. "After the 8th day bacilli become very difficult to find in the human series. In the bovine series they become increasingly numerous and very rapidly so." They conclude that "the natural resistance of the rabbit to infection with the tubercle bacillus of human type is apparently referable to a failure of this type of bacillus to multiply in the body of this species to any considerable extent. Exceptionally there are localized lesions (kidneys and lung nodules) which are associated with abundant growth and which show that this failure to multiply may be due to some positive growth restraining factor rather than to a failure of suitable nutritive materials." These authors, then, feel that the human tubercle bacillus does not grow very much in the body of the rabbit due to some undefined native restraining force.

Austin (10) approached this problem by treating rabbits inoculated intravenously with bovine and human bacilli with saline extracts of rabbit lungs. He found that fewer pulmonary lesions develop as a result of this treatment, especially in the bovine-infected group. He concludes that the "difference in the resistance of the rabbit lung to the two types of infection may be largely due to a quantitative difference in the defensive factors that are needed."

Rogers (11) studied the fate of human and avian bacilli in the liver of pigeons by histological methods after intravenous injection. He concluded that the bacilli are rapidly and extensively phagocytized by the endothelial cells lining the venous sinusoids of the liver and that the greater portion of them are rapidly destroyed by these cells in a time commensurate with the destruction of pneumococci. He found no evidence of mutiplication.

In summarizing the results obtained by cultural methods, it appears that the original localization of human or bovine tubercle bacilli in

the various organs of the rabbit follows the distribution of particulate matter (12), being greatest in the spleen, next in the liver, next in the bone marrow, and least in the kidney. The relative position of the lung among these organs is still doubtful owing to the difficulty of isolating tubercle bacilli in pure culture without treatment at the beginning of the infection. But in some work, to be described in a later communication, with 1 to 2 mg. of BCG given intravenously the deposition in the various organs of rabbits was found to be as follows: spleen 610, liver 322, lung 202, bone marrow 72 and kidney 6. These figures represent the number of colonies recovered from similar quantities of tissue after direct seeding on Dorset's medium 1 day after intravenous inoculation. They are in agreement with Bull's finding with typhoid bacilli (13).

The fate of the bacilli thus localized depends upon many factors: the dosage, whether large or small, the type, whether human or bovine, and doubtless many conditions not touched on here. Their original localization, although a factor, is far from being the controlling factor.

At first the tubercle bacilli grow and multiply in all the organs. The human type grows very fast, the bovine type much more slowly. Moreover, the rate of multiplication of both types is very different in the various organs. Thus in the spleen it is much more rapid than in the liver; it may even be negligible in the liver while very rapid in the spleen with small enough dosage, especially of bovine bacilli. But as yet in all the organs no effective opposition is offered to the multiplication of the tubercle bacillus.

Soon, however, a remarkable change takes place. The liver, spleen and bone marrow begin to destroy the tubercle bacilli. The time at which the destruction begins in these three organs depends on the dose and the type of bacillus. With large doses of human bacilli it becomes apparent between the 2nd and 4th weeks, when the bovine bacilli, in similar quantities, have reached their highest numbers. Their destruction does not become apparent until the interval between the 4th and the 8th weeks. If small quantities of human bacilli are injected, their destruction in these three organs is also delayed to the interval between the 4th and the 8th weeks.

By the 8th week after infection with a large dose of human bacilli the destruction is more complete than after a small dose. By the 8th

week after infection with a small quantity of bovine bacilli the destruction is much less complete in the spleen than after a small quantity of human bacilli. This change in the behavior of these three organs does not depend upon the actual number of bacilli accumulated in a given organ. All these facts speak clearly for the contention that the native power of the various organs to destroy tubercle bacilli is insufficient to check their growth effectually and, as a result of a stimulation most probably associated with the growth of the bacilli, an altered behavior toward the tubercle bacilli is brought about so that they are destroyed. However, while this change is sufficient in the liver and bone marrow to destroy the bacilli completely, it is insufficient to eliminate them completely from the spleen. This especially applies to small dosage.

Furthermore, the behavior of the tubercle bacilli in the lung and kidney is quite different from their behavior in the organs just mentioned. Here with large doses of human bacilli the destruction becomes apparent in the interval between the 4th and the 8th weeks instead of the interval of the 2nd to the 4th weeks as in the liver, spleen and bone marrow. With bovine bacilli in similar dosage the bacilli in the lung and kidney continue to increase in number until the death of the animal. With small doses of human bacilli the lung shows a beginning of destruction by the 8th week, when there is almost complete destruction in the liver, spleen and bone marrow. By the 4th month they have completely disappeared from the kidney and they are still decreasing in the lung. With the same dosage of bovine bacilli the organisms continue to multiply in the lungs and kidney even 4 months after infection. It is noteworthy that throughout the study it was found that after the phase of destruction has been brought about, the rate of destruction in the several organs is at first very rapid, but soon the force spends itself so that as time progresses the bacilli are more and more slowly destroyed, and a few lingering bacilli remain alive. In fact, in the spleen, 6 months after infection, a slight secondary rise was noticed. It is to be emphasized that the group of organs where tubercle bacilli are first destroyed is the liver, spleen and bone marrow, whose common function is to remove foreign particulate matter or bacteria from the body; and again, that of these three, the spleen lags behind the bone marrow and liver in its destruction of both bovine and human bacilli.

In the study cited above (1) on the cellular reaction of the organs of the rabbit toward tubercle bacilli it was found that the normal, non-immune rabbit destroyed heat-killed tubercle bacilli most effectively in the liver, less effectively in the bone marrow, much less in the spleen, and least of all in the lungs.

A correlation is thus seen between the native reaction of the several organs toward dead bacilli and the rate of destruction of living human and bovine bacilli in the same organs. The liver and bone marrow, which destroyed bacilli more effectively than the spleen in the "native" state, also destroy them more completely in the "immune" state, and these organs much more rapidly than the lung and kidney. Furthermore, the native ability of the liver and bone marrow to destroy tubercle bacilli is almost sufficient to inhibit their growth altogether in rabbits infected with small doses of both the human and bovine types, especially the liver after infection with the bovine strain. Again throughout this study it was seen that tubercle bacilli, whether human or bovine, whether in large or small doses, always grow faster in the spleen than in the liver. This is perhaps to be connected with the liver's having a greater native ability than the spleen to destroy tubercle bacilli.

These facts offer some confirmation for the hypothesis (14) that "acquired resistance is only a specific increment of natural resistance." The natural resistance in itself is always insufficient to check the growth of the bacilli in all the organs.

The acquired ability of the organs to destroy tubercle bacilli becomes increased as a result of the growth of the bacilli and the accumulations of their products in the body. The acquired ability is then added to the natural ability. In this light we can understand, to some extent at least, the fate of the bacilli in the various organs. The work of Lewis and his collaborators (15, 16) on the different hereditary powers of resistance of guinea pigs to tuberculous infection and the correlation that they have drawn between antibody production and this resistance would point in the same direction.

Römer (17) has shown that the allergic state in tuberculosis as represented by the tuberculin reaction can be brought about more quickly with larger doses of tubercle bacilli than with smaller doses. This finding has recently been confirmed by Lewis and Aronson (18).

Since it was found that the human tubercle bacilli grow faster than the bovine and that their destruction in the various organs is brought about earlier, furthermore, since with smaller doses of human bacilli the altered body reaction is brought about later than with larger doses, again, since the destruction is more complete after the same time in certain organs with large doses of both types of bacilli than with small doses, the conclusion is forced upon us that perhaps the virulence of a given type of tubercle bacillus for the rabbit is closely related to the original growth of the bacillus. This is an inverse relation. The more rapid the growth, the less virulent. For the more rapidly a given organism grows in the body the more rapidly is a resistance stimulated and the more rapidly are the bacilli destroyed. On the other hand, the more slowly an organism grows the later is this brought about. Thus in the bovine infection, while the bacilli are effectively destroyed in the liver, spleen and bone marrow, the unabated growth of bacilli in the lungs and kidney brings about the death of the animal by the replacement of tissue consequent upon the unchecked tubercle formation. Doan and Sabin (19) have recently pointed out that regression of the tuberculous process takes place in the bone marrow and, to a lesser degree, in the spleen while the disease progresses in the lung and kidney even in bovine-infected rabbits.

However, this immunity is not complete; even as late as 6 months after the infection the bacilli have not entirely disappeared from the lungs of rabbits infected with 0.001 mg. of human bacilli per kilo, and apparently they are still persisting in the spleen if not increasing.

The results obtained in the study, not yet published, of the fate of BCG in the various organs of the rabbit, are altogether in harmony with the conception that the virulence of mammalian tubercle bacilli for the rabbit is inversely related to their multiplication in the body. With these bacilli the process of quick growth followed quickly by destruction is even more rapid than with the human type.

It is pertinent to note that animals that are susceptible to the mammalian bacillus are usually more susceptible to the bovine type than to the human type as determined by experimental inoculation. Thus (20) the ox, sheep, goat, cat, pig and rabbit are much more susceptible to the bovine infection than to the human. Even the guinea pig and monkey are somewhat more susceptible to the bovine type than to

the human. The dog is apparently moderately susceptible to both, although some maintain that the dog also is more susceptible to the bovine type (21). The only species for which, it is thought, the human type is more virulent is man. But even this conclusion is doubtful (Neufeld (22)). For man is infected by the bovine bacillus largely by the intestinal route, for which much larger dosages are necessary than for the respiratory route, by which tuberculosis is far more commonly spread among human beings. It is possible, therefore, that the mechanism of virulence may be the same in all species.

CONCLUSIONS.

1. The original distribution of tubercle bacilli of both human and bovine types to the various organs of the rabbit after intravenous inoculation follows the distribution of particulate matter in the following order per gm. of tissue; spleen, liver, lung, bone marrow and kidney. The relative position of the lung amongst these organs is less certain than that of the others.

2. At first the tubercle bacilli both of the human and bovine types grow in all the organs without any effective opposition.

3. The rate of growth of both types differs in the various organs. It is much faster in the spleen than in the liver. With small doses very little growth takes place in the liver and bone marrow, especially with the bovine type.

4. The human type of tubercle bacillus grows faster in the several organs of the rabbit than the bovine type.

5. This more rapid growth of the human type is followed by an earlier and more complete destruction of the human type than of the bovine.

6. With both types destruction occurs first in the liver, spleen and bone marrow. In the lung and kidney destruction of the human type takes place later, and unchecked multiplication of the bovine type continues in these organs until the death of the animal.

7. With smaller doses of human bacilli destruction is brought about later in the liver, spleen and bone marrow than with larger doses. With both types, in a given time, destruction is more complete in some organs after a large dose than after a small dose.

8. The destruction in the various organs is rapid at first and pro-

gresses more slowly as time passes. So that even 6 months after intravenous injection of small doses of human tubercle bacilli they have not yet completely disappeared from the lung and spleen.

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AGGLUTINATION BY PRECIPITIN.

Second Paper.

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In a previous paper[1] attention was called to the fact that serum heated sufficiently to cause turbidity reacts more markedly on the addition of precipitin than unheated or slightly heated serum under the same conditions. The intensity of the reaction was explained on the basis that a portion of the proteins had been coagulated by heat and the coagulated particles had become covered with a film of undenatured proteins remaining in solution with the result that they agglutinated on the addition of antibody. Evidence confirming this view was provided by experiments in which bacteria or collodion particles were mixed with antigens and agglutinated when specific precipitin was added. It was further shown that collodion particles exposed to cow serum or crystallized egg albumin and subsequently washed until the wash fluid no longer contained the antigen agglutinated when the specific precipitin was added. Loeb[2] had previously found that collodion particles treated with proteins acquire a film of the protein on their surfaces and in consequence manifest the cataphoretic behavior of proteins. This being the case the particles employed in the writer's experiments behaved in a manner similar to suspensions of bacteria or other particulate antigens when mixed with specific antisera.

The character of the deposition on the particles is of considerable interest. From the experiments previously reported one gains the idea that the surfaces of particles exposed to protein become entirely covered with the substance. The present experiments show that this is not the case.

[1] Jones, F. S., *J. Exp. Med.*, 1927, xlvi, 303.

[2] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 395.

EXPERIMENTAL.

Since the procedure was essentially the same in all the experiments it may be said that for purposes of "sensitization" small quantities of heavy aqueous suspensions of collodion particles were mixed with antigens and incubated for various intervals. After incubation an excess of 0.9 per cent NaCl solution containing 2 cc. of N/20 NaOH per 100 cc. was added. The mixtures were then centrifuged at high speed, the supernatant discarded, and the sediment resuspended in alkaline salt solution. The process of centrifugation and washing was repeated twice and the particles were finally suspended in the alkaline salt solution. With this method relatively stable test fluids were obtained.

TABLE I.

The Effect of Antigenic Concentration on the Sensitivity of Collodion Particles as Judged by Agglutination.

Particles exposed to various concentrations of crystallized egg albumin	Dilutions of anti-egg albumin serum						Control*
	1:10	1:20	1:50	1:100	1:200	1:500	
per cent							
10	C**	C	C	++++	++	+−	−
5	C	C	C	+++	+	+−	−
2.5	C	C	C	+++	+	+−	−
1	C	C	C	++	+	−	−
0.5	C	C	++	++	+−	−	−

* Throughout the experiments the control tubes contain only suspensions of sensitized collodion particles.

** Agglutination has been indicated as follows: C = complete; ++++ marked agglutination without complete clearing of the fluid; +++ well defined deposition; ++ moderate sedimentation; + slight deposition; +− a trace of sediment.

The precipitins had been produced by the immunization of rabbits or fowls with various antigens. Some were freshly obtained, others had been on hand for several months.

Hitchcock[3] has shown that collodion membranes when in contact with solutions of crystallized egg albumin adsorb the protein on their surfaces. The amount of protein adsorbed is dependent within certain limits on the concentration of egg albumin in the solution. In the previous work reported by the writer the concentration of protein was not varied. It seemed advisable to determine first to

[3] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–27, viii, 61.

what extent the concentration of protein used for the purpose of sensitization of the collodion particles would influence subsequent agglutination. Experiment 1 was devised to throw light on this point.

Experiment 1.—To 10 cc. of 10, 5, 2.5, 1, and 0.5 per cent solutions respectively of crystallized egg albumin the same quantity of a suspension of collodion particles was added. The tubes were incubated for 1½ hours and the particles washed. After the final centrifugation the particles were suspended in alkaline salt solution and tested with anticrystallized egg albumin. The protocol in Table I is typical for such experiments. For control purposes sensitized particles were tested with normal rabbit serum, but since no agglutination occurred the results are not recorded in the table.

The antiserum agglutinated the particles in about the same titer in the first three series. This suggests that little more antigen is adsorbed by the particles from a 10 per cent solution of crystallized egg albumin than from one containing only 2.5 per cent. When the antigen concentration employed for sensitization fell below 2.5 per cent there was a slight decline in the agglutination titer although the exposure to even a 0.5 per cent solution of crystallized egg albumin resulted in well marked agglutination in the presence of specific antibody. The influence of concentration on adsorption corresponds roughly with Hitchcock's findings obtained by direct weighing of the protein taken up by collodion membranes.

On the basis of this experiment one might suppose that provided sufficient protein exists in the sensitizing fluid the particles will become completely covered and,—since an excess of protein cannot cover them more,—will react on the introduction of antiserum. Again it is possible that regardless of the concentration, provided there be sufficient protein available, the particles would adsorb only limited amounts of the antigen. Experiments dealing with this phase of the question follow.

Experiment 2.—Collodion particles were added to 5 cc. of cow serum and to a similar amount of 2.5 per cent crystallized egg albumin. The tubes were then incubated for 1 hour. After incubation an excess of alkaline salt solution was added to each tube and they were shaken and centrifuged at high speed. The supernatant was then withdrawn and the particles originally exposed to cow serum were resuspended in 5 cc. of 2.5 per cent crystallized egg albumin. The particles from the tube previously exposed to crystallized egg albumin were resuspended in

cow serum. After further incubation of 1 hour an excess of salt solution was added and the tubes were centrifuged. The particles were then washed twice by centrifuging in alkaline salt solution, suspended in alkaline salt solution, and tested for the presence of adsorbed protein by the addition of anti-cow and anti-egg albumin serum. The results are given in Table II.

Table II indicates that regardless of the order in which the particles were sensitized about the same quantity of protein was adsorbed since the particles agglutinate about as well with either precipitin. That the particles are not completely covered by the protein seems true since the reaction of the particle after sensitization to a second antigen

TABLE II.

The Effect of the Exposure of Collodion Particles to One Protein after Another.

Treatment	Tested with	Dilutions of serum						Control
		1:10	1:20	1:50	1:100	1:200	1:500	
Cow serum followed by crystallized egg albumin	Anti-cow serum	++	C	C	C	C	++	+−
	Anti-crystallized egg albumin	+++	+++	C	C	C	++	+−
	Normal rabbit serum	+	+−	+−	+−	+−	+−	+−
Crystallized egg albumin followed by cow serum	Anti-cow serum	++	C	C	C	++++	+	+−
	Anti-crystallized egg albumin	++	+++	C	C	++++	+	+−
	Normal rabbit serum	+−	+−	+−	+−	+−	+−	+−

is about the same as when this antigen was used primarily. Other experiments given later confirm these findings, indicating that the particles are not completely covered by the antigens.

It might be said that through the combination of particle and protein the latter was so changed that it would react with any antiserum. Since all the experiments are dependent on the specificity of the agglutination, an experiment which covers this phase of the question is included.

Experiment 3.—Collodion particles were sensitized with 2.5 per cent crystallized egg albumin or cow serum by the usual procedure. Both lots were then washed

in alkaline salt solution until the last wash fluid no longer contained antigen. The particles after resuspension were tested with the antiserum specific for the sensitizing substance and with other precipitins in addition. Thus particles sensitized with egg albumin were tested with anti-egg albumin, anti-cow or anti-horse serum, and those sensitized with cow serum were tested with anti-horse and anti-cow serum. The protocol is given in Table III.

The reaction must be regarded as specific since the sensitized particles react only with the precipitin specific for the sensitizing substance.

It being true that the particles are not completely covered by the antigen and that the agglutination in the presence of antibody is

TABLE III.

The Effect of Homologous and Heterologous Precipitin on Sensitized Collodion Particles.

Sensitized with	Tested with	Dilutions of serum						Control
		1:10	1:20	1:50	1:100	1:200	1:500	
Crystallized egg albumin	Anti-egg albumin	+++	++++	C	++++	++	+	−
	Anti-cow serum	−	−	−	−	−	−	−
	Anti-horse serum	−	−	−	−	−	−	−
	Normal rabbit serum	−	−	−	−	−	−	−
Cow serum	Anti-cow serum	C	C	C	++	+	+	−
	Anti-horse serum	+	+−	−	−	−	−	−

specific, it is of further interest to ascertain the effect of sensitizing the particles to several types of proteins and measuring the amount of fixation by agglutination with sera specific for the sensitizing antigen. Experiment 4 is designed to cover this phase of the question.

Experiment 4.—There were on hand five antisera and after a number of preliminary experiments it was decided to sensitize particles to the five antigens and ascertain by agglutination with the antisera to what extent the antigens had been adsorbed on the particles. The following procedure was adopted. A heavy suspension of particles was added to 5 cc. of the protein solution and incubated ½ hour. 3 or 4 volumes of alkaline salt solution was then added and the tubes centrifuged at high speed. The supernatant was withdrawn and 5 cc. of the next antigen was added to the residue and after mixing thoroughly incubated ½ hour.

This was repeated until the particles had been exposed to all five antigens. The particles were then washed and resuspended in salt solution and tested with antisera. In the protocols given in Table IV the tests were made in the order in which the particles were sensitized. For control purposes the sensitized particles were tested with normal rabbit and normal chicken serum, since immune serum from each species was employed in the experiment, but no agglutination occurred and the record is therefore omitted from the table.

It can be said that the particles adsorbed some of each protein. When the exposure was first to egg albumin and subsequently to the

TABLE IV.

The Agglutination of Collodion Particles Sensitized with Five Antigens.

Particles sensitized successively with	Tested successively with	Dilutions of antisera						
		1:10	1:20	1:50	1:100	1:200	1:500	Control
Crystallized egg albumin, rabbit serum, chicken serum, horse serum, and cow serum	Anti-egg albumin	++++	++++	++	++	+	+−	−
	Anti-rabbit serum	C	++++	++	+	−	−	−
	Anti-chicken "	++++	+++	+	+−	−	−	−
	Anti-horse "	++++	+++	+	+−	−	−	−
	Anti-cow "	++++	C	++	+	−	−	−
Cow serum, rabbit serum, horse serum, chicken serum, and crystallized egg albumin	Anti-cow serum	++++	+++	++	+−	−	−	−
	Anti-rabbit "	C	++++	+	−	−	−	−
	Anti-horse "	+++	+++	++	+	−	−	−
	Anti-chicken "	+++	++++	++	+	+	−	−
	Anti-egg albumin	++	+	+	+−	−	−	−

other antigens comparable agglutination occurred with all the antisera. If however the particles were mixed with the serum antigens first and finally treated with egg albumin, the agglutination titer indicated that little of the egg albumin was adsorbed. Other experiments indicated that this was true.

When the antigens are mixed and suspended particles are added similar but not as decisive results are obtained. This has been brought out in Experiment 5.

Experiment 5.—5 cc. of 2.5 per cent solution of crystallized egg albumin and the same amount of cow, chicken, horse, and rabbit serum were mixed and incubated

for 1½ hours with a heavy suspension of collodion particles. After incubation an excess of alkaline salt solution was added and the mixture centrifuged. The usual procedure was employed for washing and resuspending the particles. Portions of the suspension were then tested with the various antisera. The result is indicated in Table V.

It was to be expected that the fixation would be less marked when the antigens were mixed since they were diluted by each other, yet it was possible to show that some fixation actually took place between each substance and the particles. It was weakest with egg albumin and strongest with cow serum.

Since collodion particles are readily sensitized with proteins and agglutinated on the addition of specific precipitins it seemed possible

TABLE V.

The Effect of Sensitizing Collodion Particles with a Mixture of Antigens.

Antigen mixture	Tested with	Dilutions of antisera					Control
		1:10	1:20	1:50	1:100	1:200	
5 cc. each of 2.5 per cent crystallized egg albumin, cow serum, chicken serum, horse serum, and rabbit serum	Anti-cow serum	++++	C	+++	++	−	−
	Anti-egg albumin	++	++	+	−	−	−
	Anti-chicken serum	+	+++	+	−	−	−
	Anti-horse "	C	++++	++	+	−	−
	Anti-rabbit "	C	C	++	−	−	−

that they might adsorb from sera rich in antibody sufficient precipitin to agglutinate in the presence of specific antigen. A series of experiments designed to throw light on this question was undertaken. Experiment 6 is an example.

Experiment 6.—0.5 cc. of a suspension of collodion particles was added to 2 cc. of rabbit serum rich in precipitin for cow serum. The mixture was incubated 1½ hours and then 5 cc. of alkaline salt solution was added and the whole centrifuged. The sediment was washed twice more in an excess of salt solution and finally suspended in 25 cc. of alkaline salt solution. The sensitized particles were then tested (1) with antigen (cow serum); (2) with anti-rabbit serum, to determine whether or not rabbit serum protein had been adsorbed by the particles; and (3) with normal rabbit serum, for control purposes.

In addition the first supernatant fluid containing 2 cc. of the anti-cow serum and

5 cc. of NaCl solution was tested with antigen to determine whether or not the antibody content of the serum had been diminished by the admixture of the particles. For control purposes the effect of the dilution of antiserum with salt solution is included.

TABLE VI.

The Effect of Antigen on Collodion Particles Sensitized with Serum Rich in Antibody.

	Amounts of cow serum added							
	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	None
Sensitized with anti-cow rabbit serum	+	+−	+−	+−	+−	+−	+−	+−

TABLE VII.

The Agglutination of Particles Sensitized with Immune (Rabbit) Serum by Anti-Rabbit Serum.

Tested with	Amount of antiserum added						Control
	1:10	1:20	1:50	1:100	1:200	1:500	
Anti-rabbit serum (fowl)	C	C	C	++	+	+	+−
Normal fowl "	+−	+−	+−	+−	+−	+−	+−

TABLE VIII.

The Effect of the Collodion Particles on the Precipitin Titer of the Anti-Cow Serum.

	Dilutions of antigen							
	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480
Serum incubated with collodion particles	++	++	+	+	+	+	+	+
Serum diluted to the same extent but no particles added	++	++	++	+++	++	++	++	+

The effect of the addition of antigen to suspensions of collodion particles first exposed to serum rich in antibody is shown in Table VI. That there was adsorption of rabbit serum protein on the particles is brought out in Table VII; also that the particles removed some of the antibody for the immune serum is shown in Table VIII.

The experiment reported is one of a series, in others of which attempts were made to sensitize particles to anti-egg albumin or anti-horse serum and to agglutinate the particles with the specific antigen, but in no instance were they successful. It could always be shown that some of the proteins of the immune serum combined with the particles since agglutination occurred when a serum specific for the proteins was added to the suspensions of the particles. It was also possible to show that the titer of the antiserum declined after incubation with the collodion particles.

DISCUSSION.

Mention has been made of Loeb's view that the reason why collodion particles mixed with proteins behave cataphoretically like the proteins is because of the deposition of a protein film on their surfaces. The experiments reported throw more light on this phenomenon. Further proof has been brought that there is deposited on the surface of the collodion particles a portion of the protein, for the particles even when washed agglutinate in the presence of a precipitin specific for the protein. The experiments in which particles were exposed to several proteins and subsequently agglutinated with all the respective antisera argue that the deposition is not in the form of a complete film. It is probable that the protein is deposited on the surfaces in adherent patches. If this were not the case the particles would react only with a single antiserum regardless of the number of antigens applied, since if it were possible to completely layer several proteins one above another the outermost only would come in contact with the antiserum.

It is generally conceded that agglutination is a surface phenomenon dependent on a quantitative union of antigen and antibody. Relatively little has been reported on how much antigenic surface is necessary for the appearance of the phenomenon. It can be said that since collodion particles sensitized to five proteins may react by agglutination about as well to all the specific antisera, it appears that if as little as one-fifth of the surface is antigenic agglutination will occur under the proper conditions.

The experiments in which the particles were first suspended in antisera with the idea that they might adsorb sufficient antibody on

their surfaces to agglutinate in the presence of antigen are of general interest. They adsorbed antibody from the serum and furthermore proteins of the serum containing the antibody could be detected on their surfaces, yet agglutination failed to occur when antigen was added. It may be true that insufficient antibody was adsorbed. Shibley's[4] conception of bacterial agglutination, based on his experiments and those of Northrop and De Kruif and De Kruif and Northrop,[5] affords an explanation of the failure of agglutination under these conditions. He contends that agglutination depends on a specific union of antibody with antigen with the result that the cell surface is coated with a film of globulin from the immune serum. This confers upon the organism the character of denatured globulin with a reduction of the charge by electrolytes to a critical level between 12 and 14 millivolts and clumping results. A similar explanation may be applied to the agglutination of sensitized particles in the presence of antiserum specific for the sensitizing protein. The experiments in which collodion particles exposed to antiserum and then washed failed to agglutinate when antigen was added seem to indicate that the steps are irreversible, since the experimental procedure precluded the deposition of the film of denatured globulin, the necessity of which Shibley recognizes.

SUMMARY.

The fact is shown that collodion particles sensitized with various proteins adsorb sufficient protein to agglutinate in the presence of precipitin specific for the adsorbed protein. The amount of adsorption, judged by agglutination, is not dependent on the concentration of the sensitizing protein beyond a certain maximum. The agglutination resulting from the addition of immune serum to particles sensitized with protein is immunologically specific.

Particles exposed to a number of antigenic substances in succession are agglutinated by all of the appropriate antisera.

Particles exposed to immune serum and subsequently washed fail to agglutinate in the presence of antigen although some of the protein constituents of the immune serum are fixed upon them and its antibody content diminishes.

[4] Shibley, G. H., *J. Exp. Med.*, 1926, xliv, 667.

[5] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655; 1922–23, v, 127, 139, 605.

THE EFFECT OF SOME OF THE CHEMICAL CONSTITUENTS OF TUBERCLE BACILLI ON THE PROTOPLASM OF AMŒBA DUBIA.*

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PLATE 5.

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The successful isolation of various chemical constituents from tubercle bacilli by Johnson and Coghill (1) and Anderson (2, 3) has afforded an opportunity of using these substances in biological experiments. Recently Sabin and Doan (4) have tested the chemical fractions on rabbits and found distinctly different reactions to these components on the part of the clasmatocytes and monocytes.

The action of the derivatives from the tubercle bacilli on *Amœba dubia* was studied with the micrurgical technique, the same procedure being used that has been described previously (5). Although there is no evidence that fresh water amebæ are associated with tubercle bacilli, the use of such material was considered desirable because the ameba represents a simple cell upon which many data have been gathered in this laboratory with respect to its reaction to various chemicals.

Immersion Experiments.

Protein.—Amebæ were immersed in suspensions of proteins 304, 304-A (from unautoclaved, defatted organisms), 304-B (from autoclaved, defatted organisms), 308, and Dr. Florence B. Seibert's culture medium protein.[1] These suspensions were brought with NaOH to a hydrogen ion concentration varying from pH 7 to pH 8.2, depending

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[1] The author wishes to express his gratitude to Drs. Treat B. Johnson, R. J. Anderson, and Florence B. Seibert for materials, and to Dr. William Charles White for his cooperation.

upon their maximum solubility. As controls, suspensions of serum albumin were used. Amebæ, placed in such suspensions, and kept in the ice box, showed no change in motility, in ability to produce pseudopodia, or in longevity, compared to amebæ kept under normal cultural conditions for at least 5 days. This innocuous effect of the proteins is true even though some denaturation occurs. If the containers are permitted to remain at room temperature and the proteins become more quickly and completely denatured the amebæ lose their pseudopodia, their plasmalemmæ become stiff, and they become very sluggish.

Phosphatides.—Amebæ were immersed in emulsions of the phosphatides A-3 and A-4. The resulting hydrogen ion concentration varied between pH 5.5 and 6. Amebæ immersed in emulsions of A-3 died in 1 day in concentrations of 0.01 per cent, in 2 days in 0.003 per cent, and were not able to exist in a normal state for 5 days until a dilution of 0.0015 per cent was reached. In A-4 amebæ died in 3 days in a 0.006 per cent emulsion and were living and well for at least 5 days in a 0.003 per cent emulsion.

The manner of death of amebæ in the phosphatide emulsions is characteristic of that due to surface-dispersing action, such as is caused by soaps (6). A normal ameba (Fig. 1) is characterized by a distinct plasmalemma, varying degrees of pseudopodial formation, and, as judged by the rate of the movement of the granules, an actively flowing endoplasm and a more quiescent ectoplasm. When amebæ are placed in emulsions of phosphatide derived from tubercle bacilli, the plasmalemma becomes sluggish and unable to flow even though the cytoplasm is in active motion. The cell, therefore, forms no pseudopodia and becomes round. Gradually the plasmalemma loses its form, breaks in places, and disappears as though it were dissolved, leaving the naked protoplasm as solidified debris (Fig. 2).

With emulsions of lecithin and of lecithin and cholesterol as controls no evidence of toxicity was seen when amebæ were immersed in as strong a mixture as 0.06 per cent, which is the strongest emulsion compatible with visibility of the amebæ in such a thick suspension.

Fatty Acid.—The fatty acid derived from the phosphatide is immiscible with the aqueous medium containing amebæ. If, however, amebæ are brought to an adjacent drop of fatty acid with the micro

needle, the plasmalemma of the cell which is in contact with the fatty acid quickly disappears.

If the ameba is pushed back into the water the plasmalemma is reformed. In one case an ameba was snared rapidly out of the aqueous medium into the fatty acid droplet and the plasmalemma disappeared instantly. When this acid is dissolved in 95 per cent alcohol, it gives an indication of having a pH between 2.8 and 3.0.

Polysaccharide.—Amebæ immersed in a 1 per cent aqueous solution of the polysaccharide A-8 (pH 7 with NaOH) derived from tubercle bacilli show no toxic effects even after 4 days. The growth of fungi makes longer observations impossible.

Injection Experiments.

Protein.—Injections of moderately large amounts ($\frac{1}{3}$ of an ameba full) of suspensions of 304 and 308 render the amebæ sluggish for about 5 minutes. Smaller amounts ($\frac{1}{4}$ of an ameba full) have only slight effect and larger injections ($\frac{1}{3}$–$\frac{1}{2}$ of an ameba full) usually cause quiescence and death. Injections of serum albumin and of Dr. Seibert's culture medium protein were relatively without effect upon the amebæ. Fig. 3 shows an ameba injected with protein 304. All the granules stand out distinctly, indicating almost complete quiescence, as contrasted with the blur seen in the normal ameba represented by Fig. 1, indicating rapid movement during the time of photographic exposure.

Phosphatides.—Injections of phosphatide emulsions have no other effect than that seen if the solvent alone is injected.

Fatty Acid.—This substance is not readily miscible with protoplasm. When injected it forms a discrete sphere in the cytoplasm. Usually the ameba relegates the droplet to the rear and appears to be in the process of pinching it off with the surrounding protoplasm. Before this can be accomplished, however, the plasmalemma in the vicinity of the drop disappears. If a sufficiently large drop of fatty acid is injected, the plasmalemma of the entire ameba can be made to dissolve. If the nucleus approaches the drop, it becomes hyaline. In one case the nucleus flowed into the fatty acid droplet and became hyaline.

Polysaccharide.—Injection of as strong a solution of polysaccharide

A-8 as 1 per cent in large amounts had no more effect upon the ameba than injection of water.

DISCUSSION.

The action of the chemical constituents derived from tubercle bacilli upon amebæ may give little indication of the exact physiological responses of other cells to these bodies. But the general effect of these bacterial substances on any particular part of the ameba gives a suggestion of the probable chemical reaction involved. It is interesting to note that the phosphatide and fatty acid act upon the surface of the cell and the protein affects the interior. The polysaccharide has no apparent action on the normal ameba.

One function of the apparently non-toxic polysaccharide, as far as its chemical significance is concerned, may be to make the fatty acid miscible with protoplasm when these two substances are combined as a phosphatide. What other functions, either chemical or immunological, the polysaccharide may have is beyond the scope of these experiments.

It is of interest to consider the possible relation this work may have to the action of the whole organism on the cell. If the phosphatide fraction is in the waxy outer coat of the organism and this is miscible with the plasma membrane of the cell, it is easy to conceive how the bacillus can penetrate into the cell. If the outer covering of the organism is digested once the bacterium is engulfed, the protein may be free to act on the internal protoplasm.

In these experiments there is no direct evidence that the substances used have some specific effect by virtue of their derivation from the tubercle bacillus. As a matter of fact, except for their slower rate of action, the phosphatide and the fatty acid act as all plasma membrane solvents such as soaps (6), CO_2, or lactates (7). There is some evidence that the proteins 304 and 308 are more toxic upon the internal cytoplasm than serum albumin or culture medium protein. But Dr. Seibert (8) finds that her protein gives a 5+ skin reaction as contrasted to a 3+ with 304. This question of specificity of action, therefore, needs further investigation. These experiments aim merely to indicate the general chemical reactions of the derivatives used upon a simple cell.

CONCLUSIONS.

1. Protein fractions derived from tubercle bacilli are toxic to the interior of *Amœba dubia* but have no action on the plasmalemma.

2. Phosphatide fractions dissolve the plasmalemma but have no effect on the internal cytoplasm.

3. The fatty acid fraction has a marked solvent action on the plasmalemma when brought in contact with the surface of the cell. When injected it may slowly penetrate the cytoplasm to dissolve the contiguous plasmalemma.

4. The polysaccharide fraction has no effect upon the surface membrane or upon the internal cytoplasm of *Amœba dubia*.

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EXPLANATION OF PLATE 5.

FIG. 1. A normal ameba. Blur in center indicates rapidity of motion of endoplasm; ectoplasm moving more slowly; distinct plasmalemma.

FIG. 2. Effect of immersion of amebæ in phosphatide emulsion. Lower ameba rounded, plasmalemma quiescent and beginning to disappear, internal cytoplasm active. Upper ameba, plasmalemma dissolved, dead debris left.

FIG. 3. Effect of injection of protein fraction into ameba. Quiescence of internal cytoplasm.

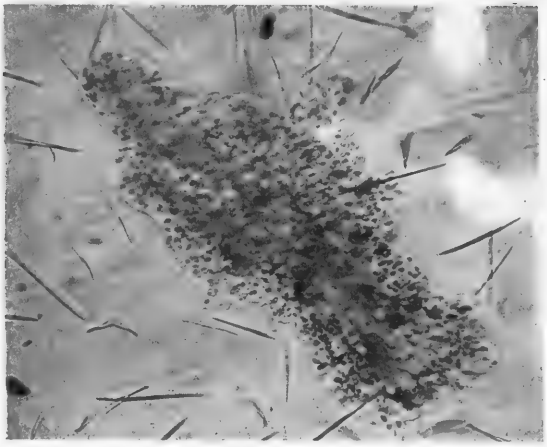

FIG. 1.

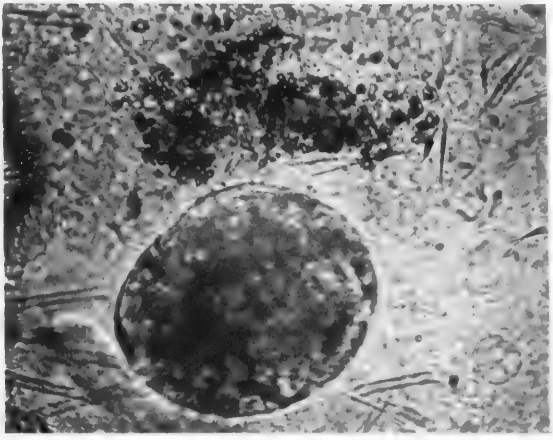

FIG. 2.

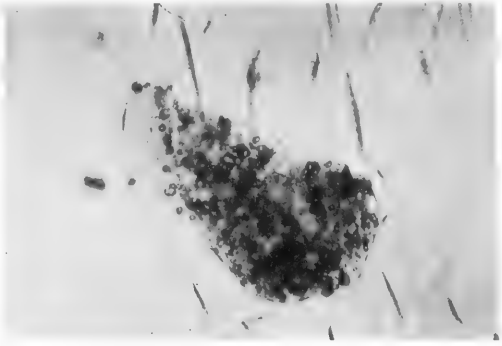

FIG. 3.

(Reznikoff: Chemical constituents of tubercle bacilli.)

THE RELATION OF STREPTOCOCCI TO HERPES VIRUS ENCEPHALITIS.

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It is generally believed at the present time that no convincing evidence has been brought forward in support of a definite bacterial incitant of epidemic and of herpes virus encephalitis.

Recently, Evans and Freeman[1] have described an amphophilic, aerobic, pleomorphic, green-producing streptococcus which they obtained from nasal washings, the heart blood, and the mesencephalon of a patient who died during an acute attack of epidemic encephalitis. This organism appeared in tubes of chopped meat medium inoculated with the washings, or a few drops of heart blood, or with small pieces of mesencephalon. It grew readily upon ordinary media in subplants and did not ferment salicin, raffinose, mannitol, or inulin. Litmus milk was curdled.

The microorganism grew at various times as a long chain streptococcus, a diplococcus, a spore-bearing rod, a giant coccus, or as a diphtheroid. Of these, the streptococcus was pathogenic in rabbits when inoculated into the brain, and produced a symptom-complex associated with pathological findings which were thought to resemble those of epidemic encephalitis in man.

In a continuation of these studies, Evans[2] reported that she was able to isolate the pleomorphic organism from rabbit brains containing one or another of six different strains of herpes virus. Brains carrying respectively Strains J. B.[3] and H. F.,[4] from The Rockefeller Institute, were among these specimens. Chopped meat medium inoculated with 1 to 2 cc. of a 10 per cent saline suspension of infected brain was used. In three instances the organism was procured directly from glycerolated brain material, while in the case of the other three specimens, it proved necessary for positive cultivation to secure fresh brain material by animal passage. The bacteria were thought to be identical with those isolated by Evans and Freeman from epidemic encephalitis in man.

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[1] Evans, A. C., and Freeman, W., *Pub. Health Rep., U. S. P. H.*, 1926, xli, 1095.

[2] Evans, A. C., *Pub. Health Rep., U. S. P. H.*, 1927, xlii, 171.

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

[4] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

The ease with which the organisms were cultivated by the authors mentioned here led us to a study of their source and their relation to virus encephalitis in the rabbit. Cultivation experiments have been made with rabbit brains which contained either the J. B.,[3] the H. F.,[4] or the Levaditi[3,5] strains of encephalitogenic virus.

EXPERIMENTAL.

Media.—The following media were used in the cultivation experiments.

Chopped meat medium, prepared according to the directions given by Evans.[2] "Ordinary beef infusion broth is prepared and the hydrogen ion concentration is adjusted to pH 8.0. Instead of discarding the meat from which the broth is made, the ground meat particles are placed in tubes to a depth of about 1 inch. Sterilization is at 15 pounds for 1½ hours. During sterilization the hydrogen ion concentration is reduced to about pH 6.8."

Dextrose beef infusion broth containing 1 per cent dextrose.

5 per cent rabbit's blood-beef infusion agar in Petri dishes.

Culture Technique.—Cultures were made not only of brains carrying respectively the three strains of encephalitogenic virus, but of normal brain tissue, of chopped meat particles, and of dextrose broth. In each test, 1 cc. of a 10 per cent saline suspension of a ground tissue or of broth was inoculated into three to six tubes of fresh medium. The broth, like the tissue, had been ground as such in a sterile mortar under a hood, with precautions for sterility.

Cultures from Normal and Virus Encephalitis Brain Material.

The results obtained with brain material are summarized in Table I. Cultures were made with herpes virus-infected rabbit brains placed for varying periods of time in sterile 50 per cent glycerol, fresh normal, and fresh virus-infected brains from rabbits and guinea pigs, and fresh brain material of guinea pigs inoculated intracerebrally with non-infective cerebral tissue.

From the table it will be noted that irregular results were obtained. A single culture of *Staphylococcus aureus* was obtained from one of the glycerolated specimens of virus-infected brains, and from the fresh virus-infected brains a streptococcus in one instance and a large coccus in another. Two strains of streptococci were isolated from normal guinea pig brain material and two from the brain tissue of guinea pigs which had been previously inoculated subdurally with

[5] Levaditi, C., and Harvier, P., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 354; *Ann. Inst. Pasteur*, 1920, xxxiv, 911.

the Levaditi strain of virus. All positive cultures were procured from liquid media, the blood agar plates remaining sterile in all instances.

Cultures of Chopped Meat Medium and of Broth.

It was important to ascertain whether the meat particles used in the medium contained organisms which had resisted sterilization.

TABLE I.

Results of Cultures.

Animal	Virus strain	Status at death	Condition of brain	Growth in chopped meat medium	Growth on blood agar
Rabbit A	Levaditi	Typical virus encephalitis	Glycerolated 8 days	*Staphylococcus aureus* in 1 tube	Negative
" B	"	" "	Fresh	Negative	"
" C	"	" "	Glycerolated 11 days	"	"
" D	"	" "	Glycerolated 13 days	"	"
" E	H. F. 1	" "	Glycerolated 90 days	"	"
" F	J. B.	" "	Glycerolated 113 days	"	"
" G	Levaditi	" "	Fresh	Streptococci in 1 of 3 tubes	"
" H	"	" "	"	Large cocci in all tubes	"
Guinea Pig A	H. F. 1	" "	"	Negative	"
" " B	J. B.	" "	"	"	"
" " C	Levaditi	Normal	"	"	"
" " D	"	"	"	Streptococci in 1 of 3 tubes	"
" " E	"	"	"	" "	"
" " F	Control	"	"	Negative	"
" " G	"	"	"	"	"
" " H	"	"	"	Streptococci in 1 of 3 tubes	"
" " I	"	"	"	" "	"

Three uninoculated lots of meat medium, containing 260 tubes, prepared in the usual manner and at different times, were incubated from 3 days to 3 weeks at 37°C. The tubes were examined frequently for bacterial growth and were found to be sterile. As a further check, the meat particles from twelve tubes were smeared upon the surface of blood agar plates. These also remained sterile.

The next experiment concerned the possibility of obtaining organisms from ground meat particles and dextrose broth.

The meat particles from each of twenty-three tubes of uninoculated chopped meat medium were ground in a mortar with the usual precautions against contamination. 1 cc. of the resulting suspensions was inoculated into fresh tubes of meat medium and dextrose broth respectively, and upon blood agar plates. Six tubes of dextrose broth free from growth after 2 weeks incubation, were used to provide control material. From each of these, 4 cc. was removed and "ground" in a sterile mortar. This material was seeded into dextrose broth and upon blood agar plates.

Streptococci were obtained in culture from 6 of the 23 tubes from which the meat particles were removed and ground, diphtheroids from 6, spore-producing rods from 9 (together with streptococci in one case), giant cocci from 14, hay bacilli from 1, and *Staphylococcus albus* from 5. A streptococcus was isolated from one tube seeded with the "ground" dextrose broth. The blood agar plates were sterile.

Description of the Streptococci.

The streptococci procured in the way just mentioned grew readily in chopped meat medium, in dextrose broth, and upon rabbit blood agar plates. The growth was granular in the chopped meat medium and in dextrose broth. Small colonies having a greenish tinge and a small zone of hemolysis appeared on rabbit blood agar plates. Both short and long chain types developed in the liquid media, and often long chains of parallel rows of diplococci. Dextrose, saccharose, maltose, and lactose were fermented, while mannitol, xylose, salicin, inulin, and raffinose were not fermented. The reaction to Gram's stain was variable. The organisms showed no marked pleomorphism, and there was no suggestion of a change to a giant coccus, rod form, or diphtheroid.

Intracerebral Inoculation into Rabbits.

The virulence of eight strains of streptococci isolated as above described was tested by intracerebral injection into rabbits.[6] Two

[6] All experiments on animals were made with the aid of complete ether anesthesia.

rabbits were used for each strain. Four of these strains failed to cause death of the rabbits. Both the animals inoculated with a fifth died of a purulent meningoencephalitis, and so too did one rabbit out of each pair inoculated with the remaining three strains.

The following protocol is typical of the findings in infected animals.

Rabbit A was inoculated intracerebrally with 0.35 cc. of an 18 hour initial dextrose broth subplant of a streptococcus isolated from ground meat particles. 18 hours after injection, the rabbit's temperature rose to 105.2°F. It was weak, had frequent generalized involuntary muscular contractions, and a Parkinsonian type of head tremor. The pupils were widely dilated, the breathing slow and labored, and a marked urine retention was present. The animal became moribund within 24 hours and was etherized.

The brain, removed with sterile precautions, was markedly congested and soft. The cerebrospinal fluid was increased in amount and contained numerous fibrin flakes. A smear from the brain showed diplococci; and pure cultures of streptococci were isolated from it as also from the heart blood. Microscopically the brain showed diffuse polymorphonuclear infiltration of the meninges, large hemorrhages and small abscesses in the cortex, generalized edema, and a perivascular infiltration with polymorphonuclear neutrophiles and lymphocytes. In addition, a generalized polymorphonuclear neutrophilic infiltration of the brain tissue was noted. No intranuclear inclusion bodies were found.

Diagnosis: Purulent meningoencephalitis.

Experiments on Immunity.

Rabbits which had proved resistant to intracranial inoculation of the streptococci were subsequently reinoculated subdurally with active herpes virus. All died from virus encephalitis.

Six rabbits were injected corneally with an initial culture of streptococci obtained from the brain of a rabbit dying from streptococcic meningoencephalitis. No keratitis developed, such as is produced by all three of the strains of encephalitogenic virus used in the experiments. 2 weeks later, these six animals were reinoculated in the cornea with the Levaditi strain of virus. All but one rabbit (an animal discarded from another experiment) developed a characteristic keratitis with fatal termination from virus encephalitis.

No cross-immunity between streptococci and encephalitogenic virus was demonstrated in these experiments.

Differential Glycerolation.

It is not known definitely how long bacteria will survive in glycerolated brain material, kept at +4°C. Herpes virus survives indefinitely under such conditions. The brains of rabbits which had died of herpes virus encephalitis but which contained streptococci, as the cultures showed, were placed in 50 per cent glycerol for 120 and 133 days respectively. At the end of these periods it proved impossible to recover streptococci from the glycerolated brain tissue, in chopped meat medium, dextrose broth, or upon blood agar plates. Yet the same glycerolated brain material, inoculated intracerebrally, produced typical virus encephalitis in rabbits. In the brains of the animals that died no streptococci were found.

SUMMARY AND CONCLUSIONS.

Cultures of microorganisms similar to those described by Evans have been obtained in media inoculated with suspensions of herpes virus-infected brains prepared by grinding. But they have also been isolated from saline suspensions of uninoculated meat particles ground in a sterile mortar, and from dextrose broth treated in the same way. It is believed that these organisms are contaminants introduced during the process of grinding. Since they enter the material in no great number, one may suppose them to be suppressed by animals inoculated with the ground substance. In artificial media, on the other hand, they find favorable conditions for multiplication. In our experience, no growth of microorganisms is obtained in routine cultures of virus-infected brains, when fragments, instead of ground material, are used—a fact which may be taken to support the explanation just given.

The tests of the part played by streptococci in experimental virus encephalitis failed to disclose that the microorganisms have any etiological relationship to the affection. The intracerebral injection of rabbits with the cultures procured in the course of the experiments produces a purulent type of meningoencephalitis which does not resemble virus encephalitis either in its symptom-complex or in its pathology. The same type of meningitis follows the injection of streptococci derived from ground meat particles, from "ground" broth, from normal brains, and those infected with herpes virus. Some

rabbits manifested resistance to the streptococci, whereas all that have been inoculated intracerebrally with the three strains of herpes virus used in this study have proved susceptible thereto. Certain of the rabbits just mentioned which had proved resistant to streptococci inoculated into the brain or cornea were injected with herpes virus and reacted typically. Comparative tests have revealed that the streptococci are more sensitive to the destructive effect of 50 per cent glycerol than is herpes virus. From all this, it can be concluded that streptococci are not the visible form of herpes virus, nor do they produce in rabbits effects like those induced in the brain and cornea by the herpes virus.

STUDIES ON BLOOD CELL METABOLISM.

I. The Effect of Methylene Blue and Other Dyes upon the Oxygen Consumption of Mammalian and Avian Erythrocytes.*

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(Received for publication, May 16, 1928.)

Although a considerable number of studies have been published on various phases of the metabolism of blood cells, the relative importance of fermentation and of respiration in their normal activities is still very imperfectly understood. Still more obscure is the metabolic behavior of pathological blood cells, although it may be of considerable significance. It is of particular interest, for example, to examinethe activities of the cells of leucemic blood in the light of the recent advances made in our knowledge of the metabolism of cancer cells.

A question which at once attracts attention in this field is that of the cause of the great variations which are found in the respiration of various types of normal erythrocytes. Several years ago it was shown by one of us (1) that normal human erythrocytes, separated from leucocytes and blood platelets by appropriate methods, have a scarcely demonstrable oxygen consumption when incubated at 38°C. for some hours under sterile conditions. The utilization of oxygen was considerable, however, when large numbers of reticulocytes were present in the blood, such as are found in hemolytic jaundice or in other conditions with active blood regeneration. These findings in general were in harmony with the previous studies of Morawitz and Itami (2) upon the oxygen consumption of anemic blood, and of Warburg (3), who found, using the Barcroft-Haldane manometer, that although normal adult mammalian erythrocytes in general had a very low oxygen consumption, the respiration was much increased in the blood of

* The second paper of this series will appear in a forthcoming number of *The Journal of Biological Chemistry*.

young rabbits and appeared to be roughly proportional to the amount of polychromasia present. In contrast to the slight oxygen consumption of non-nucleated mammalian erythrocytes, Warburg showed that the nucleated erythrocytes of birds (geese) have a very large oxygen consumption.

By the use of the technique previously described (1), but with the more accurate Van Slyke constant volume blood gas apparatus now available, it can be shown that non-nucleated adult mammalian erythrocytes have an exceedingly small, but measurable oxygen consumption when incubated at 38°C. This is accompanied by the production of carbon dioxide.

The interesting question now arises as to the cause of this difference in the metabolism of mature mammalian erythrocytes, in contrast to that of the young or immature forms and that of the nucleated cells of avian blood. To state that the oxidative activities of the different types of red blood cells seem to be related in some intimate way to the presence or absence of nuclear material furnishes no satisfactory clue as to the actual mechanism involved. It is conceivable that in the course of its development some inhibiting substance is formed in the mammalian erythrocyte which almost stops the respiratory metabolism of the cell. On the other hand, it is also conceivable that in the course of development, some essential link in the respiratory mechanism is lost in the cell so that oxidations no longer take place. We wish to present evidence for the latter view. We have found that the oxygen consumption of non-nucleated mammalian erythrocytes can be greatly increased, and can approach the magnitude of that of the nucleated cells of avian (goose) blood when one of a number of vital dyes, and particularly methylene blue in low concentration (0.005 to 0.0005 per cent), is added to the blood before its incubation.* The amount of oxygen absorption when compared to that in ordinary defibrinated blood may be increased many times. This rise occurs in the blood of all mammals tested—dog, beef, sheep, and man, and is accompanied by a corresponding evolution of carbon dioxide (Table I).

* The methylene blue used in these experiments was Purified Methylene Blue from Leopold Cassella and Co., Frankfurt a.M.

TABLE I.

The Effect of Methylene Blue upon the Respiration of Mammalian Erythrocytes.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed
	vol. %	*vol. %*	*vol. %*	*vol. %*	*per cent*
1. Man—defibrinated blood					
Control before incubation	36.79	19.24			
after incubation	38.57	18.94	1.78	0.30	1.6
+M.B. after incubation	48.01	6.82	11.22	12.42	64.6
2. Man—washed cells suspended in Locke's solution					
Control before incubation	4.22	17.01			
after incubation	4.57	16.53	0.35	0.48	2.8
+M. B. after incubation	14.64	3.39	10.42	13.62	80.1
3. Dog—defibrinated blood					
Control before incubation	13.91	23.20			
after incubation	19.45	22.77	5.54	0.43	1.9
+M.B. after incubation	22.17	12.64	8.26	10.56	45.5
4. Dog—defibrinated blood					
Control before incubation	9.99	27.95			
after incubation	19.35	27.80	9.36	0.15	0.05
+M.B. after incubation	25.00	14.40	15.01	13.55	48.5
5. Sheep—defibrinated blood					
Control before incubation	21.64	16.32			
after incubation	26.92	16.10	5.28	0.22	1.5
+M.B. after incubation	32.17	7.94	10.53	8.38	51.4
6. Beef—defibrinated blood					
Control before incubation		22.62			
after incubation		22.03		0.59	2.6
+M.B. after incubation		12.84		9.78	43.3
7. Dog*—defibrinated blood					
Control before incubation	13.59	22.01			
after incubation	15.74	20.02	2.15	1.99	9.2
+M.B. after incubation	21.35	10.09	7.76	11.92	54.1

The incubation time in all of these experiments was 3 hours at 37°. Methylene blue (M.B.) 0.005 per cent.

* Blood taken from a dog that had been previously subject to large bleedings. Examination of the blood for immature forms was not made.

TABLE I—*Concluded.*

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed
	vol. %	*vol. %*	*vol. %*	*vol. %*	*per cent*
8a. Man—defibrinated blood					
Control before incubation	11.11	20.45			
after incubation	16.47	19.47	5.36	0.98	4.8
+M.B. after incubation	25.60	11.32	14.49	9.13	44.6
8b. Man—washed cells of above (8a) suspended in Locke's solution					
Control before incubation	5.28	19.95			
after incubation	5.92	19.24	0.64	0.71	3.6
+M.B. after incubation	12.80	10.93	7.52	9.02	45.2
9. Man†—washed cells suspended in Locke's solution					
Control before incubation	2.94	22.95			
after incubation	5.30	20.35	2.36	2.60	11.3
+M.B. after incubation	13.68	8.44	10.74	14.51	63.4
10. Dog—washed cells suspended in Locke's solution					
Control before incubation	3.36	22.58			
after incubation	4.35	21.62	0.99	0.96	4.3
+M.B. after incubation	13.56	9.58	10.20	13.00	57.6

† Blood from a patient with polycythemia vera. A good deal of polychromasia and some immature cells present. It may be seen that in this experiment the percentage acceleration of respiration on the addition of methylene blue is much less than with normal human erythrocytes.

The blood used in these studies was freshly drawn and carefully defibrinated with glass beads or a stirring rod. It was then passed with very gentle suction once or twice through a column of several centimeters of sterile cotton wool. The filtration was carried out in the cold (10°C.) to prevent glycolysis. This proved a most effective method of removing the leucocytes, as demonstrated by numerous control counts, which rarely exceeded 1000 per c. mm. The blood was then thoroughly saturated with oxygen and a control sample taken for immediate gas analysis. A portion was then treated with the dye or other reagents whose effect was to be studied, and another portion, untreated, was taken for a control. These samples were incubated at once either under a thick layer of paraffin oil, or, when carbon dioxide analyses were also desired, in small stoppered bottles, provided with a long side arm capillary, essentially as described in the earlier paper(1). They contained a few glass beads and were well paraffined. Certain control analyses were also done by means of a microrespiration apparatus.

The microrespiration apparatus used was slightly modified from the design of

that described by Winterstein (4). Our vessels were made in the shape of tiny Erlenmeyer flasks with very broad bases. Two small chambers were blown out from the vessel wall at opposite sides to carry other fluids, whose effect on the respiration it was desired to test. In the present instance these were methylene

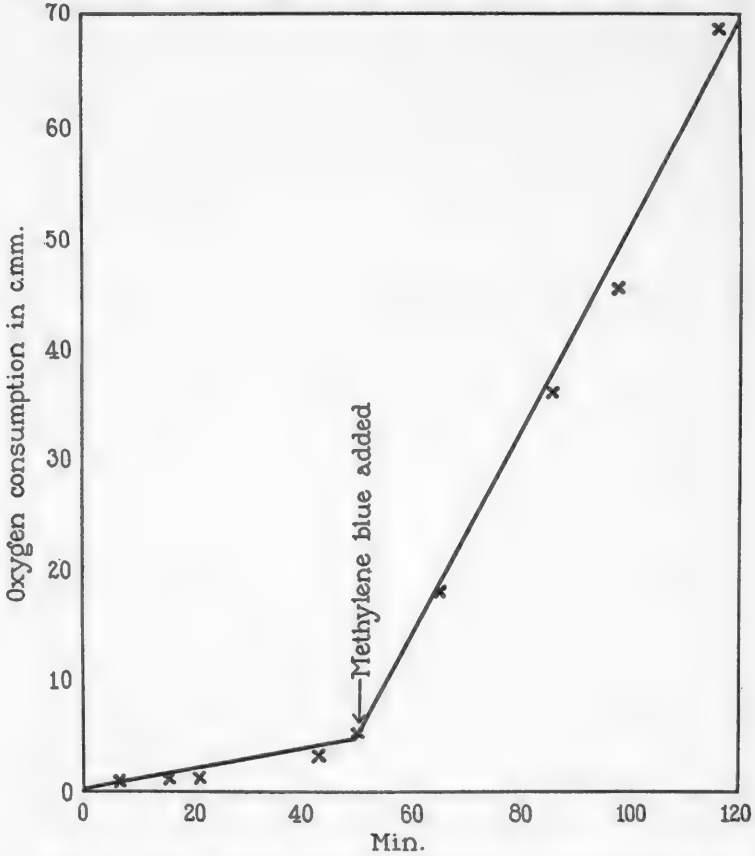

CHART 1. Microrespiration experiment. The effect of methylene blue upon the oxygen consumption of human erythrocytes (1.0 cc.).

blue or other dyes, and potassium cyanide, or other agents which inhibit cell oxidations. A special shaking device was constructed, and the apparatus was shaken at a rate of 75–100 per minute. A large rubber bulb with set screw attachment was used to regulate the mercury burette level. When two essential

precautions were observed—absolute cleanliness of the capillary tubes which contained the bubble, and care in the maintenance of a constant temperature of the water bath—the apparatus proved very useful in our hands. It presents certain advantages over the Barcroft-Warburg method in that elaborate calibration and calculations are avoided, since the oxygen consumption is found by direct readings on the mercury burette.

The increased oxygen consumption of human erythrocytes upon the addition of methylene blue in the microrespiration apparatus is indicated in the experiment shown in Chart 1.

We have excluded the possibility of methemoglobin formation in these studies by spectroscopic examination and particularly by a

TABLE II.

The Effect of Temperature upon the Oxygen Consumption of Mammalian (Dog) Erythrocytes.

	Oxygen content before incubation	Oxygen content after 3 hrs. incubation	Oxygen content after 24 hrs. incubation
	vol.%	*vol.%*	*vol.%*
Control at 21°C.	19.45	19.20	18.84
+M.B. 0.05%		17.40	13.92
Control at 38°C.		18.80	12.72
+M.B. 0.05%		9.60	Completely reduced

Methylene blue (M.B.) concentration 0.005 per cent.

second estimation of the oxygen capacity of the blood after incubation, upon resaturation with air or oxygen. The latter is the more reliable test. Methylene blue alone gives a prominent line between the characteristic bands of methemoglobin in the spectroscope which may readily cause confusion on casual inspection.

The recent study of Wales, Munch and Schwartze (5) is of interest in regard to the question of methemoglobin formation. The authors made intravenous injections into rabbits and cats of very large amounts of certain dyes used for the coloring of foods and then made spectrophotometric determinations upon blood samples withdrawn at various intervals. With all of the dyes studied, reduced hemoglobin was formed within an hour. Complete reduction occurred when the specimens were permitted to stand for 24 hours or more. No evidences of methe-

moglobin formation could be detected in any of their studies. It seems likely that these dyes produced an effect similar to that of methylene blue upon the respiration of the erythrocytes.

The respiration takes place in fresh red blood cells carefully washed with Ringer's solution at approximately the same rate as in defibrinated blood alone and the increased respiration on addition of methylene blue is of about the same order of magnitude for both as is indicated in Experiments 8a and 8b, Table I.

The accelerated oxygen consumption in the presence of methylene blue proceeds even at room temperature, but its optimum appears to be at 37°C. (Table II).

TABLE III.

The Effect of Varying the Methylene Blue Concentration upon the Respiration of Mammalian Erythrocytes.

Nov. 21—dog blood	Before incubation		After incubation		Output carbon dioxide	Consumption oxygen
	Content carbon dioxide	Content oxygen	Content carbon dioxide	Content oxygen		
	vol. %	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*
Control—no M.B.	17.75	29.25	19.86	28.93	2.11	0.32
M.B. 0.005%			29.88	14.45	12.13	14.80
M.B. 0.0025%			30.00	14.21	12.25	15.04
M.B. 0.001%			28.75	14.80	11.00	14.45
M.B. 0.0005%			27.98	18.06	10.23	10.19

Methylene blue (M.B.) concentration 0.005 per cent.

The final concentration of methylene blue used in these studies has been 0.005–0.0005 per cent. Higher concentrations produced no marked increase in the respiration and at 0.0005 per cent concentration there was a diminution in the effect of the added methylene blue (Table III).

It is known that potassium cyanide, even in very dilute solution, stops cell oxidations. We have therefore studied the effect of potassium cyanide upon methylene blue respiration in the experiments presented in Table IV. It will be seen that the increased oxygen consumption upon the addition of methylene blue was nearly as great in the presence of the cyanide as in the untreated control. The effect

of methylene blue upon the respiration of mammalian blood cells is not therefore inhibited by this concentration of potassium cyanide (N 0.001).

The effect of methylene blue upon the active respiration of the nucleated red blood cells of birds was found to be entirely different from the effect which it had on the non-nucleated mammalian red blood cells. As is indicated in Table V and in the microrespiration experi-

TABLE IV.

Effect of Potassium Cyanide upon Methylene Blue Respiration.

	Content oxygen		Content oxygen
	vol. %		*vol. %*
Beef			
Blood before incubation	22.61	After 3 hrs. incubation	22.03
Blood + N/5000 KCN		After 3 hrs. incubation	21.95
Blood + 0.005% methylene blue		After 3 hrs. incubation	12.84
Blood + N/5000 KCN + 0.005% M.B.		After 3 hrs. incubation	12.74
Dog			
Blood before incubation	23.80	After 3 hrs. incubation	23.40
+ N/5000 KCN		After 3 hrs. incubation	23.58
+ 0.005% methylene blue		After 3 hrs. incubation	14.63
+ N/5000 KCN + 0.005% M.B.		After 3 hrs. incubation	15.10
Man			
Blood before incubation	19.42	After 3 hrs. incubation	19.18
+ N/1000 KCN		After 3 hrs. incubation	19.09
+ 0.005% methylene blue		After 3 hrs. incubation	9.42
+ N/1000 KCN + 0.005% M.B.		After 3 hrs. incubation	11.70

ment shown in Chart 2, the respiration of goose erythrocytes, either from normal blood or from animals rendered anemic by bleeding, is increased by methylene blue, but to a much smaller extent than in the case of non-nucleated mammalian cells. Whereas in the latter the respiration is accelerated by methylene blue on the average twenty to fiftyfold, in avian blood it is never increased more than twice that of the simple defibrinated blood to which dye has been added.

Chart 3 illustrates the results obtained from a microrespiration experiment and shows that the respiration of goose erythrocytes which

TABLE V.

The Effect of Methylene Blue upon the Respiration of Avian (Goose) Erythrocytes.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed	Time of incubation	Condition of animal
	vol. %	*vol. %*	*vol. %*	*vol. %*	*per cent*		
1. Dec. 6—defibrinated blood							
Control before incubation....	11.59	21.60					
after incubation.....	18.77	11.63	7.18	9.97	46.2	3 hrs.	Normal
+M.B. after incubation.....	30.98	1.01	19.39	20.59	95.5		
2. Dec. 16—defibrinated blood							
Control before incubation....	12.00	16.55					
after incubation.....	20.46	7.84	8.46	8.71	52.6	1½	Anemic
+M.B. after incubation.....	24.28	1.36	12.28	15.19	91.6	hrs.	
+T.B. after incubation.....	23.51	1.76	11.51	14.79			
3. Feb. 1—defibrinated blood							
Control before incubation....	22.64	11.24					
after incubation.....	26.20	7.05	3.56	4.19	37.2	40	Anemic
+M.B. after incubation.....	28.42	5.03	4.78	6.21	55.2	min.	
4. Feb. 6—defibrinated blood							
Control before incubation....	22.40	22.00					
after incubation.....	32.05	16.92	9.65	5.08	23.1	40	Normal
+M.B. after incubation.....	35.50	14.16	13.10	7.84	35.6	min.	
The blood for Experiments 1, 2, and 3 was taken from the same animal during the course of several large periodical bleedings.							
5. Dec. 21—washed cells in Locke's solution							
Control before incubation....	4.00	19.65					
after incubation.....	10.66	11.35	6.66	8.30	42.2		
+M.B. after incubation.....	12.66	8.44	8.66	11.21	57.1		Anemic

M.B. means methylene blue 0.005 per cent solution.
T.B. means toluylene blue 0.005 per cent solution.

has been inhibited by the addition of potassium cyanide may be again restored when methylene blue is added.

We have extended our studies to an examination of the effect of

several other dyes whose oxidation-reduction potentials have been determined, including Bindschedler's green, indigo disulfonate, *o*-chlorophenol indophenol, phenol indophenol, and toluylene blue. Some

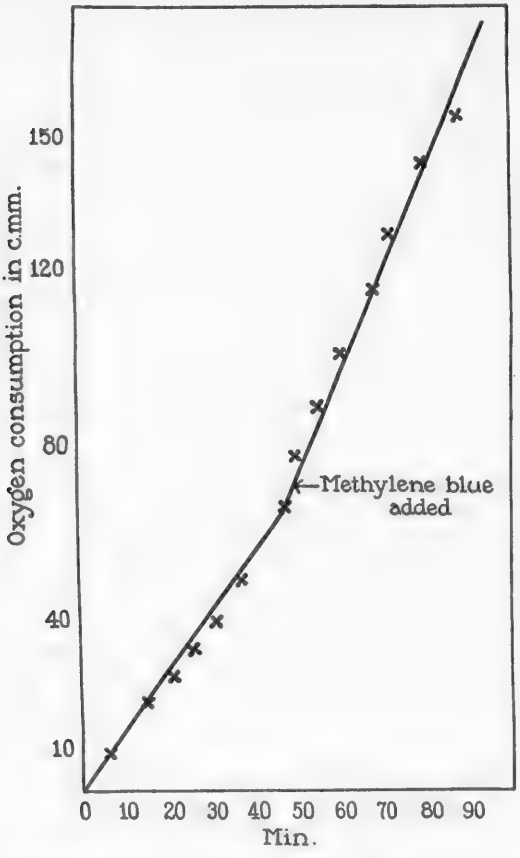

CHART 2. Microrespiration experiment. The effect of methylene blue upon the oxygen consumption of goose erythrocytes.

of these were kindly supplied to us by Professor W. Mansfield Clark. As seen in Table V, Experiment 2, and in Table VI, none of these with the exception of toluylene blue is as effective as methylene blue in

stimulating respiration at this range of concentration. The fact that no direct correlation can be shown between the oxidation-reduction potential of these dyes and the kinetic phenomena involved in the

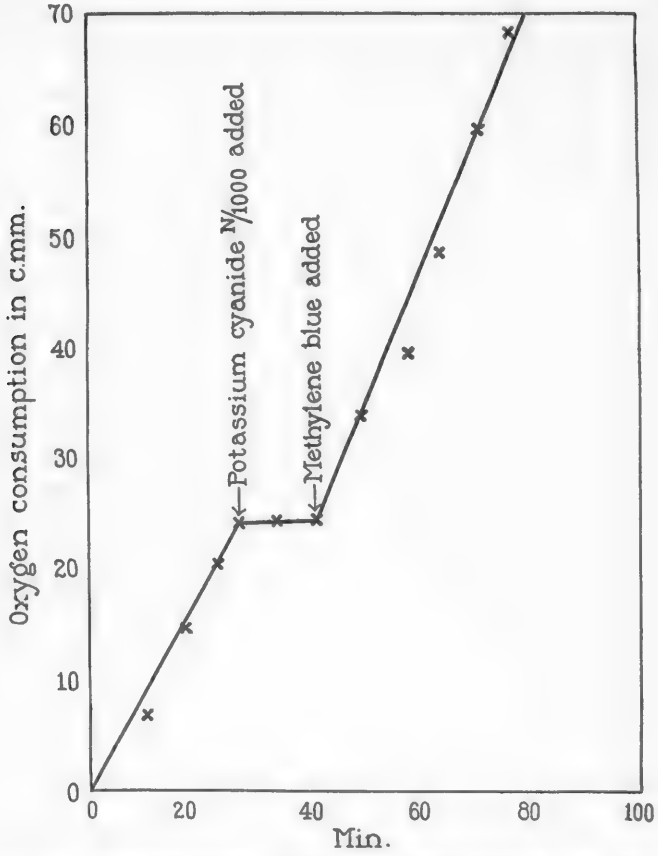

CHART 3. Microrespiration. The effect of methylene blue upon the oxygen consumption of goose erythrocytes in the presence of potassium cyanide.

accelerated cellular oxidations is not surprising. The question of the variable permeability of the cell membranes to the several dyes and the possibility of their variable toxic action must be taken into considera-

tion. It is at least of some significance that the potentials of the dyes which activate these oxidations fall within a rather restricted range.

The increased red blood cell metabolism reported in the present paper recalls the work of Heymans and Heymans (6), who produced fever in dogs ranging as high as 43° or 44°C., together with a marked increase in the output of carbon dioxide, by the intravenous injection of methylene blue, 0.05–0.10 per kilo of body weight.

TABLE VI.

The Effect of Various Dyes upon the Respiration of Mammalian Erythrocytes.

	Oxygen content before incubation (A)	Oxygen content after incubation (3 hrs. 38°C.) (B)	Oxygen consumption (A–B)	Percentage oxygen consumption $\frac{A-B}{A} \times 100\%$
	vol. %	*vol. %*	*vol. %*	
Oct. 28—beef blood; 3 hrs. incubation				
Control blood alone	18.50	18.11	0.39	2.1
Methylene blue 5 mg. % added		11.70	6.80	36.8
Indigo disulfonate 5 mg. % added		17.75	0.75	4.1
o-Chlorophenol indophenol 5 mg. % added		16.45	2.05	11.1
Phenol indophenol 5 mg. % added		16.28	2.22	12.0
Toluylene blue 5 mg. % added		10.00	8.50	46.0
Dec. 21—goose blood; 1 hr.'s incubation				
Control blood alone	19.65	11.35	8.30	42.2
Methylene blue 0.0025%		8.44	11.21	57.1
Bindschedler's green 0.0025%		9.04	10.61	54.0
Dec. 22—human blood (polycythemia vera); 3 hrs. incubation				
Control blood alone	23.58	22.35	1.23	5.2
Methylene blue 0.0025%		8.44	15.14	64.2
Bindschedler's green 0.0025%		15.52	8.06	34.2

In seeking an explanation of the effect of methylene blue upon erythrocyte respiration our attention was drawn to the studies reported by Meyerhof (7) upon staphylococci, precipitated by acetone in the cold, dried over phosphorus pentoxide, and subsequently heated *in vacuo* at 100–107°C. Under such circumstances, and by proper selection of the heating time, he was able to suppress to a large extent the oxygen absorption of this material. Upon the addition of methyl-

ene blue, 2.5 or 5 mg. per cent, the oxygen utilization again increased, although never to the same extent as in the unheated material. The methylene blue, by reason of its oxygen carrying properties was considered to take the place of some component of the respiration system which had been destroyed by the previous manipulations. The usual inhibiting effect of potassium cyanide upon the oxygen consumption of the killed staphylococci was much less marked in the presence of the methylene blue. The respiratory quotient lay within physiological limits —0.6 to 1.0.

Meyerhof reports a similar effect upon the oxygen consumption of acetone yeast, although here the optimal concentration of methylene blue was higher (0.02 — 0.1 mg. per cent). It was found to be unnecessary to heat the acetone yeast because the respiration of the unheated yeast, after the acetone-ether treatment, had already fallen to about 2 per cent. The respiration of the living cells, which are much less affected by methylene blue, was not inhibited in the presence of 0.0015 N KCN when 0.05 per cent methylene blue was added. Acetone yeast respiration in the presence of methylene blue is only affected to the extent of 10–20 per cent even in the presence of 0.01 N KCN. Macerated yeast juice (Lebedew) was found to behave in a similar manner in the presence of methylene blue.

The oxidation mechanism in the experiments which we report in the present paper appears to be analogous to that encountered by Meyerhof in his experiments. The effect of methylene blue occurs even in the presence of cyanides in both sets of experiments. The rate of oxidation in the mammalian erythrocytes in the presence of methylene blue is increased to about the rate of oxygen consumption found in the untreated nucleated erythrocytes of birds. Mammalian erythrocytes can be regarded as old or altered cells, in which oxidation processes have been largely suppressed through the disappearance of some link of the oxidation system. This link, however, is present in the younger cells. Methylene blue appears to be able to take the place of this substance and to act essentially as an oxygen carrier enabling the erythrocytes to utilize in their metabolism the oxygen stored in them as oxyhemoglobin. The respiration then resumes a rate similar to that present in the nucleated erythrocytes of avian blood.

The demonstration of proper respiratory quotients in studies on the

metabolism of blood has been difficult. Most authors content themselves with the statement that "physiological" respiratory quotients are obtainable, but actual figures are seldom given. At the time of our previous paper (1) we were unable to obtain agreement in this regard between oxygen consumption and carbon dioxide production in human blood. Consequently no figures on the carbon dioxide

TABLE VII.

The Respiratory Quotient of Mammalian and Avian (Goose) Erythrocytes with and without the Presence of Methylene Blue. Washed Cells Suspended in Locke's Solution.

	Content carbon dioxide (A)	Content oxygen (B)	Carbon dioxide output (C)	Oxygen consumption (D)	Respiratory quotient $\frac{C}{D}$
	vol. %	*vol. %*	*vol. %*	*vol. %*	
*Man**					
Control before incubation	4.22	17.01			
after incubation	4.57	16.53	0.35	0.48	0.73
+M.B. after incubation	14.64	3.39	10.42	13.62	0.77
Dog†					
Control before incubation	3.36	22.58			
after incubation	4.35	21.62	0.99	0.96	1.00
+M.B. after incubation	13.56	9.58	10.20	13.00	0.79
Goose‡					
Control before incubation	4.00	19.65			
after incubation	10.66	11.35	6.66	8.30	0.80
+M.B. after incubation	12.66	8.44	8.66	11.21	0.78

M.B. means methylene blue 0.005 per cent.

* Experiment 2 Table I.

† Experiment 10 Table I.

‡ Experiment 5 Table V.

production were published. The difficulty in metabolism studies upon incubated mammalian erythrocytes lies in the excessive production of carbon dioxide compared to the oxygen consumption, as may readily be observed by an inspection of Table I. A large initial formation of carbon dioxide in shed blood appears to occur in the serum in the presence of the red cells. It does not occur when the cells are

washed and suspended in Locke's solution (Table I, Experiments 8a and 8b). The amount of extra carbon dioxide thus produced in the 1st hour of incubation may amount to 2 millimols per 100 cc. of blood. The source of this extra carbon dioxide is under investigation. As seen from Table VII, the respiratory quotient of both avian and mammalian erythrocytes usually lies between 0.75 and 0.80 both in the presence of methylene blue and in its absence, when the serum is removed and the cells are suspended in Locke's solution.

TABLE VIII.

Effect of Hemolysis (Freezing and Thawing) upon Oxygen Consumption of Goose Blood.

	Oxygen content before incubation	Oxygen content after incubation	Oxygen consumption	
	vol. %	*vol. %*	%	
Control blood	16.55	7.84	52.6	1 hr.
Control blood + 0.005% M.B.		1.76	89.4	incubation

After hemolysis by freezing and thawing, the material was centrifuged at high speed and the top and bottom layers separated. The top material contained only a small amount of cellular debris and no nuclear fragments when examined microscopically. The bottom layer contained cell fragments and nuclei and a very few intact erythrocytes.

Top layer control	10.84	10.40	4.0	2 hrs.
Top layer control + 0.005% M.B.		9.13	15.8	incubation
Bottom layer control	13.67	10.90	20.3	2 hrs.
Bottom layer control + 0.005% M.B.		7.94	41.9	incubation

Effect of Destruction of the Cell Membrane (Freezing and Thawing) upon Oxygen Consumption and the Effect of Methylene Blue.

Warburg has shown that the cell membranes of erythrocytes (goose) can be destroyed by freezing and thawing. When the material is then centrifuged at high speed, two layers may be obtained, the top containing only hemolyzed cell contents, while the lower contains nuclear material and the remains of the formed elements. He found that the respiratory activity was then confined to the lower layer which contained the nuclear material. We have repeated these experiments,

and have found (Table VIII) that the oxygen consumption is greatly reduced in both top and bottom layers, as compared to the consumption in untreated defibrinated blood. Although the oxygen consumption of the material in the top layer is much less than it is in the bottom layer, it is still appreciable. The addition of methylene blue produces the usual increase in oxygen consumption in both layers, but it is interesting to note that the effect is much more marked in the very slowly respiring top layer which contains no nuclear material.

CONCLUSIONS.

1. The respiratory metabolism of non-nucleated mammalian erythrocytes is enormously accelerated and approaches the magnitude of the metabolism of the nucleated erythrocytes of birds on the addition of methylene blue (and certain other dyes), to a final concentration of 0.005–0.0005 per cent.

2. In the presence of methylene blue the respiration is accelerated even when M/1000 KCN is also present.

3. The accelerated respiration due to methylene blue occurs at room temperature but it is most active at 38°.

4. Methylene blue in the above concentration accelerates the respiration of avian (goose) erythrocytes to a much smaller extent than it does the respiration of the erythrocytes of mammalian blood, while the effect upon anemic goose blood seems to be less than it is upon cells of normal goose blood.

5. Owing to a rather large initial carbon dioxide formation in defibrinated blood on incubation, which may not be related to the immediate respiratory process, proper respiratory quotients cannot be obtained in whole blood. When the cells are separated from the serum and suspended in Locke's solution, respiratory quotients are obtained upon incubation comparable to those of other resting mammalian cells, as well as of the actively respiring erythrocytes of birds.

6. The hypothesis is advanced that methylene blue acts in the rôle of an oxygen carrier, supplying a substance which has disappeared from adult mammalian non-nucleated erythrocytes and restoring their metabolic activity to an extent comparable to that of the young immature forms, or to that of the actively respiring avian (goose) blood.

We wish to acknowledge the kindness of Professor Michaelis in drawing our attention to the analogy between our findings and those reported by Meyerhof.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND.

III. The Effect of Injections of Epinephrine on the Hemolysin Formation in Normal Rats.

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In the previous studies on normal and suprarenalectomized rats, it was found that suprarenalectomy results in an early depression in hemolysin forming capacity during several weeks following the operation (1, 2). Traumatization of the perisuprarenal tissue had the same effect on hemolysin formation as suprarenalectomy. In traumatizing the perisuprarenal tissue the nerve and vascular supply of the suprarenal gland are undoubtedly severely injured. It has been thoroughly established by the work of Dreyer (3), Tscheboksaroff (4), and Stewart and Rogoff (5) and others that the output of epinephrine from the suprarenal gland is controlled by a nervous mechanism, and that interruption of the nervous pathway results in a diminution of the epinephrine output. In this connection, it is of interest to determine the influence of epinephrine injections on the antibody formation in normal and suprarenalectomized rats.

Josue and Paillard (6) found that injections of epinephrine had no influence on opsonin formation. Hrma (7) was unable to observe an effect from injections of epinephrine on the hemolysin titer or complement content of the serum of rabbits though Pinner (8) noted a decrease in the complement titer of guinea pig serum. Bijlsma (9) did not succeed in varying the agglutinin or hemolysin forming capacity of normal and unilaterally suprarenalectomized rabbits by epinephrine injections. Borchardt (10) noted an increase in typhoid agglutinins in human beings following the injection of epinephrine subcutaneously administered 20 days after the injection of the typhoid vaccine. Oda (11) likewise observed an acceleration of the typhoid agglutinin formation following injections of epinephrine in human beings. The bactericidal action of serum of rabbits following the subcutaneous or intraperitoneal injections of epinephrine is increased. Large doses of epinephrine, however, exert an opposite effect (12).

Method.

In all the experiments recorded Mulford's "adrenin" was used (1/1000). Dilutions were made in physiological saline and all injections were administered subcutaneously. The daily amounts used for each rat were brought up to a total volume of 1 cc. with physiological saline and this quantity was administered in two injections of 0.5 cc. each at intervals of 6 hours. All the rats used in the experiments recorded in this communication were normal adult albino rats approximately 4 months old. Titers were determined 5, 8, 11, and 14 days subsequent to the injection of the antigen. The antigen employed in all instances was a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of sheep cells.

In determining the titer of hemolysin present in the serum, progressive dilutions of the rat serum to be tested were made. .1 cc. of a 5 per cent suspension of sheep cells and 2½ units of complement were added. The total volume of each tube was brought up to 1 cc. with physiological saline. The tubes were incubated at 37° during 30 minutes and readings were made immediately. Traces of hemolysis equivalent to a one plus or lesser reading on a scale in which four plus represents complete hemolysis, were disregarded. That dilution in which hemolysis was partial but definite, that is, a two plus reaction, was read as the titer of the hemolysin present in the serum.

In the first group of experiments the daily quantity of adrenin injected was kept constant but the number of days that the adrenin was administered prior to and subsequent to the injection of the sheep cells varied. Thus three series were used in this group. All rats in this group received 0.4 mg. of adrenin per kilo per day in two doses. In all instances a 24 hour period was allowed to intervene before and after the injection of sheep cells during which period no adrenin was given. This was done to eliminate any possible immediate influence of epinephrine on the absorption of antigen into the circulation. The first series was given injections of adrenin during 3 days prior and 4 days subsequent to the injection of the antigen. The second series received the injections only during 4 days subsequent to the injection of antigen. The third series of the first group was injected only during 1 day prior and 2 days subsequent to the injection of sheep cells.

In the second group of experiments, the number of injections was kept constant but the daily quantity injected was varied. Thus all rats in this group received injections of adrenin during 3 days prior and 4 days subsequent to the injection of sheep cells. One series, however, received 0.08 mg. per kilo per day or one-fifth the quantity employed in the first group of experiments. A second series received daily injections of 0.02 mg. per kilo per day or one-twentieth the quantity. A third series received 0.01 mg. or one-fortieth, a fourth series, 0.006 mg. or one-sixtieth the amount used in the first group of experiments.

In all series control rats were injected with equivalent volumes of physiological saline in two daily injections. Hemolysin formation in normal rats was also studied.

TABLE I.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.4 Mg. per Kilo per Day in 2 Doses, Administered during 3 Days Prior and 4 Days Subsequent to the Injections of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total* daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	*mg.*						
256	260	0.1	7	14	800	Died		
257	220	"	"	"	0	0	0	
258	240	"	"	"	80	800	400	
259	190	"	"	"	30	200	160	60
260	195	"	"	"	200	300	200	100
261	280	"	"	"	0	200	40	
290	250	"	"	"	160	300	300	100
291	215	"	"	"	600	300	300	160
292	200	"	"	"	160	160	300	160
293	290	"	"	"	400	Died		
294	280	"	"	"	1,600	800	1,600	100
296	190	"	"	"	60	80	Died	
360	150	0.06	"	"	400	360?	80	
361	140	"	"	"	80	300	Died	
362	160	"	"	"	150	400	Died	
363	170	"	"	"	400	800	0	
364	190	"	"	"	4,000	2,000	60	
365	190	"	"	"	800	600	0	
			Saline controls					
397	240	1 cc.	"	"	6,000	Died		
262	190	"	"	"	4,000	400	Died	
356	220	"	"	"	6,000	3,000	200	200
398	190	"	"	"	12,000	4,000	160	
298	230	"	"	"	12,000	2,000	1,000	80
299	200	"	"	"	6,000	1,000	1,000	200
366	225	"	"	"	1,600	800	80	
367	230	"	"	"	4,000	4,000	100	
			Normal rat					
297	240	—	—	—	12,000	4,000	4,000	400
295		—	—	—	2,000	300	300	200

* Total daily volume brought up to 1 cc. with physiological saline.

TABLE II.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.4 Mg. per Kilo per Day in 2 Doses, Administered during 4 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total daily amt. of adrenin	No. of days adrenin injected	No of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	*mg.*						
340	140	0.06	4	8	200	—	100	200
341	155	"	"	"	40	40	40	40
342	155	"	"	"	400	160	20	40
343	140	"	"	"	400	160	60	20
344	170	"	"	"	1,600	400	300	200
345	160	"	"	"	3,000	1,600	600	—?
			Saline controls					
346	140	1 cc.	"	"	4,000	400	80	80
347	140	"	"	"	400	80	0	0
			Normal rats					
264	200	—	—	—	6,000	1,600	300	60
265	210	—	—	—	18,000	6,000	2,000	200

TABLE III.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.4 Mg. per Kilo per Day in 2 Doses, Administered during 1 Day Prior and 2 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	*mg.*						
330	150	0.06	3	6	16,000	3,000	600	200
331	145	"	"	"	16,000	1,600	200	160
332	145	"	"	"	8,000	1,000	200	200
333	160	"	"	"	16,000	3,000	400	200
334	140	"	"	"	8,000	600	100	80
335	150	"	"	"	4,000	600	60	20
			Saline controls					
336	140	1 cc.	"	"	32,000	4,000	800	300
			Normal controls					
338	140	—	—	—	32,000	4,000	1,000	200
339	150	—	—	—	2,000	100	10	10

I. The Effect on Hemolysin Formation of Subcutaneous Injections of Epinephrine in Amounts of 0.4 Mg. per Kilo per Day.

In the first series the epinephrine was administered during 3 days prior and 4 days subsequent to the injection of sheep cells. In this series 28 rats were used, of which 18 were treated with epinephrine, 8 were saline controls, and 2 normal controls. The hemolysin formation in the epinephrine treated rats was markedly depressed in 17 of the

TABLE IV.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.8 Mg. per Kilo per Day in 2 Doses, Administered during 3 Days Prior and 4 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	*mg.*						
350	260	0.02	7	14	6,000	8,000	400	400
351	255	"	"	"	2,000	2,000	200	Died
352	250	"	"	"	800	800	160	160
353	260	"	"	"	800	600	160	30
354	255	"	"	"	800	1,000	60	20
355	258	"	"	"	600	200	40	160
			Saline control					
356	255	1 cc.	"	"	6,000	3,000	200	200
			Normal control					
359	258	—	—	—	2,000	—	300	200

18 rats. One gave a normal titer. The average initial titer in these rats* was 1/328 as compared to the average titer of normal rats of 1/7000. The saline control rats gave normal titers. This depression was strikingly consistent throughout the experiment. The most marked depression was noted in this series.

In the second series the same daily quantity of epinephrine was given during 4 days subsequent to the injection of the antigen. Ten rats were used, of which 6 were treated with epinephrine, 2 were saline controls, and 2 normal rats. The average titer of the treated

* A single normal reading of 1/4000 was omitted in this average. Including this exception, the average titer of the series is 1/551.

rats was 1/940 or still definitely lower than the normal or saline control rats.

The third series of the first group received the same large daily injections of epinephrine but only during 1 day prior and 2 days subsequent to the injection of the sheep cells. Nine rats were used, of which 6 were injected with epinephrine, 1 with saline solution, and 2 were normal rats. All gave high normal titers.

TABLE V.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.02 Mg., 0.01 Mg., and 0.006 Mg. per Kilo per Day in 2 Doses, Administered during 3 Days Prior and 4 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Amt. of adrenin per kg.	Total daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
						5th day	8th day	11th day	14th day
	gm.	*mg.*	*mg.*						
385	230	0.02	0.005	7	14	1,600	300	100	40
386	225	"	"	"	"	12,000	4,000	800	200
387	260	"	"	"	"	10,000	Died		
388	260	"	"	"	"	1,000	800	100	40
389	250	0.01	0.0025	"	"	3,000	Died		
390	275	"	"	"	"	8,000	4,000	100	60
391	220	"	"	"	"	1,600	2,000	400	100
392	230	"	"	"	"	6,000	2,000	160	40
393	280	0.006	0.0015	"	"	1,600	1,000	60	
394	220	"	"	"	"	2,000	800	160	40
395	225	"	"	"	"	4,000	Died		
396	220	"	"	"	"	4,000	2,000	600	100
				Saline controls					
397	225	1 cc.	—	"	"	6,000	Died		
398	222	"	—	"	"	12,000	4,000	160	

II. The Effect on the Hemolysin Formation of Varying Quantities of Epinephrine, Administered during 3 Days Prior and 4 Days Subsequent to the Injection of Sheep Cells.

The first series received 0.08 mg. adrenin per kilo per day or one-fifth the amount used in the first group of experiments. Eight rats were used, of which 6 were injected with epinephrine, 1 with saline

solution, and 1 was a normal rat. The average titer of the treated rats was 1/1800 or slightly less than normal. The depression was less marked than when the larger amounts were used.

The next three series were run in a single experiment. Fourteen rats were used. Four received daily injections of 0.02 mg. per kilo, 4, 0.01 mg. per kilo, 4, 0.006 mg. per kilo. There were 2 saline controls. The titers in all instances, when these smaller amounts were used, were normal.

DISCUSSION.

The pharmacological effects of injections of epinephrine have been the subject of extensive experiments. Very little, however, is known of its influence on the phenomena of immunity. We have noted a marked depressant effect on antibody formation of repeated injections of large quantities of epinephrine administered for several days prior and several days subsequent to the injection of antigen in normal adult albino rats. When smaller quantities were administered the same number of times, the depression was less marked. The same daily quantities administered during a shorter period depressed the titer to a less degree. The range of effective amounts of epinephrine was determined. With small quantities there is little effect or perhaps a slight rise above the normal. The quantity of epinephrine injected in the first group of experiments produced a glycosuria, which persisted during 12 hours following the injection. The rats reacted to this quantity of epinephrine by extreme restlessness followed by a short period of depression. In some instances the rats were depressed from the start and dragged their hind legs about as if they were paralyzed. This, however, wore away in a few minutes.

In a recent study on the relation of the reticulo-endothelial system to the glands of internal secretion, Leites and Riabow (13) noted a depression in the phagocytic power of the reticulo-endothelial cells following an injection of a large amount of epinephrine. This was determined by the rapidity of disappearance from the circulation of sugar of iron (ferri oxidum saccharatum) injected into the blood stream of rabbits. They contend that large amounts of epinephrine exhaust these cells. If the lymphoid and reticular elements play a

rôle in antibody formation,—and considerable evidence to this effect has already accumulated,—it is possible to explain the action of large amounts of epinephrine on antibody forming capacity in this manner.

The opposite effect noted by some observers using a single injection of a small amount of epinephrine does not in any way contradict this interpretation. We have not exhausted the possible methods of administrating epinephrine and it is possible that we shall find in some instances, when small amounts are used, a slight stimulating effect.

It has been found that removal of the suprarenals results in a depression in hemolysin forming capacity during the early period following the operation (2). The administration of large quantities of epinephrine in normal rats has a similar effect. In both instances there is apparently an exhaustion of the antibody forming mechanism.

SUMMARY.

The effect of subcutaneous injections of varying amounts of epinephrine on the hemolysin formation of normal adult albino rats was studied. In one group of experiments the rats received 0.4 mg. per kilo per day in two injections during 3 days prior and 4 days subsequent to the injection of sheep cells. Another series received the same daily amounts but only during 4 days subsequent to the injection of antigen. A third series received the same daily amounts only during 1 day prior and 2 days subsequent to the injection of sheep cells. In a second group of experiments smaller amounts of epinephrine were given during 3 days prior and 4 days subsequent to the injection of antigen. The amounts used were 1/5th, 1/20th, 1/40th, and 1/60th the daily quantity of epinephrine injected in the first group of experiments.

CONCLUSION.

Large amounts of epinephrine injected repeatedly before and after the injection of antigen depress the antibody forming capacity of normal adult albino rats to a marked degree. The depression in antibody formation is roughly proportional to the quantity of epinephrine injected. Small amounts have no detectable effect on antibody formation.

We wish to acknowledge our appreciation to Dr. David Marine for his suggestions and criticisms during the progress of this work.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

VI. Conduction of High Frequency Currents through the Living Cell.

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In this paper an attempt will be made to correlate the investigations of biophysicists with those questions which are of fundamental importance in a study of diathermy. A survey of the literature shows that the intimate relationship of these fields has not been appreciated. Biophysicists on the one hand have paid little attention to the phenomenon of heat production with the passage of high frequency currents, while those studying diathermy have neglected the problem of cellular conduction and penetration. It is obviously of great importance to know whether the diathermy current passes through the living cell, and if so, what are the effects produced.

The only constant effect which is known to be produced by high frequency alternating currents is that of heat production. The chemical effects of electrolysis disappear as the alternations exceed a frequency of 5,000 to 10,000 per second (1). The explanation for this fact lies in the rapidity of the reversal of the current which does not allow the chemical action of one phase to manifest itself before it is neutralized by the opposite phase (2). The absence of any exciting influence on the tissues through which the current passes is probably analogous to this absence of chemical effect. If the high frequency current is rectified so that it is not alternating in character, electrolysis and muscle excitation will be produced, even if the frequency of interruptions be as high as 500,000 per second (3).

The complexity of living tissue makes determinations extremely difficult of the actual course and means of transportation of the current through it. Philippson (4), Fricke and Morse (5), and McClendon (6), in their work on muscle, liver, and blood cells, have shown that as the frequency of alternations is increased the impedance of the living cell decreases, meaning by impedance the sum of all the various hindrances to the passage of the current. At a frequency of 10^5 to 10^7 cycles per second a point is reached where the impedance of living and dead cells is in close agreement. Beyond this frequency, according to these authors, the

current passes in part at least through the cell, and not wholly through the extracellular fluid, as was formerly supposed. The red blood cell is surrounded by a very efficient dielectric layer, equal in thickness to a layer composed of approximately 20 to 30 carbon atoms, and allowing little or no penetration of ions. The interior of the cell has a comparatively low resistance, equivalent to a 0.17 per cent NaCl solution. The cell, therefore, acts as a condenser, which transmits the current by its capacitance. In the living body the various fascial layers also act as dielectrics (7) of varying degrees of efficiency.

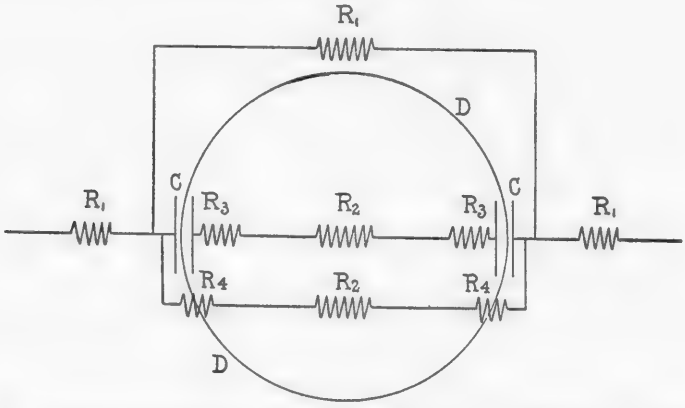

FIG. 1. Diagrammatic representation of the passage of high frequency currents through the living cell.

R_1—Resistance of extracellular fluid.
R_2—Resistance of cell interior.
R_3—Dielectric loss.
R_4—Condenser leak.
C —Capacitance of cell.
D —Dielectric layer surrounding cell.

It will be realized that the passage of high frequency currents through the living body is an extremely complex phenomenon. The whole system consists of resistances and capacitances, both in parallel and in series, some of which are stationary and some in motion. Fig. 1 represents in diagram the passage of the current through a single unit of this system—namely, the living cell. Even this representation is, of course, too simple, since the various phases in the colloidal system we call protoplasm must influence the passage of the current within

the cell (8, 9) and the protoplasm itself is constantly changing in conductivity (10).

One would expect these general principles to be applicable to the diathermy current. However, there are several differences between the currents used by the investigators mentioned and those used in diathermy which might render such an application fallacious. The diathermy current consists of pulses of impressed discontinuous oscillatory trains which probably contain many harmonics. This type of current is not necessarily comparable to a high frequency current of the pure sine wave form. Also, impedance measurements with currents of low and high voltage are not necessarily comparable (11).

There are two characteristics of the passage through living cells of low voltage high frequency alternating currents of the pure sine wave form. These, as described by the authors referred to, manifest themselves in the fact that the impedance is not changed when the cell is broken down by lysis, and in the fact that with increase of cellular concentration the impedance increment is less than that found with continuous currents.

The experiments described in this paper were planned for the purpose of finding out whether these two characteristics hold true with the currents used in diathermy.

EXPERIMENTAL.

Details of the apparatus used to produce the high frequency alternating current have been described elsewhere (12). The oscillatory current generated by the discharge of the condenser across the spark gap is of high voltage but low amperage. Owing to the high decrement of the circuit the oscillations are in the form of a series of discontinuous trains, each train being damped down before the next train commences. The frequency is 1.25×10^6 but the oscillations are not of the pure sine wave form, as harmonics are probably present.

Since it is impossible to make any accurate impedance measurements by the bridge method with this type of current, we were forced to approach the subject from a somewhat different angle from that which has been adopted by the investigators just mentioned. The experiments about to be described naturally fall into two groups, more or less independent of each other. They will, therefore, be described separately.

I. Direct Measurement of Heat Production in Blood and Serum.

The heat produced in blood and serum by the passage of a known quantity of current was measured by placing the blood or serum in a specially constructed glass cylinder, so arranged that temperature measurements could be made while the current was passing through it.

The material used was defibrinated sheep blood, obtained fresh from the slaughter house. The cell volume of the blood was varied by centrifugalization. Hematocrit readings were made at 2,000 revolutions per minute for 45 minutes. The cylinder was 20 cm. long and 6.5 cm. in diameter, with a capacity of 640 cc. It was closed by two cork stoppers equipped with circular lead-tin electrodes, the leads passing through the corks. Three openings on the long axis of the cylinder allowed the introduction of thermometers and the fluids to be examined. The cylinder was insulated against heat loss by wrapping it with thick felt. With this apparatus it was possible to measure the temperature changes in blood during the passage of high frequency currents through it.

To correct for heat loss during the current flow, measurements were made on the rate of cooling of water contained in the cylinder, with various differences between the temperature of the water and room temperature. When this difference amounted to 10°C. the rate of cooling was 0.04°C. per minute, showing that insulation against heat loss was fairly efficient. In the experiments to follow, however, corrections for this heat loss were made.

The strength of the current in all the experiments was 400 milliamperes, as recorded by the hot wire milliammeter and the time of exposure to the current was 10 minutes. By altering the spark gap, the milliammeter reading was kept constant throughout each experiment, so that, although the true amperage was probably changing owing to the changes of temperature in the various circuits (13), the amount of current which passed through each individual sample was approximately the same. To standardize as far as possible these changes in temperature, both the apparatus and the sample to be examined were at room temperature when each experiment began. In spite of these precautions, preliminary experiments with salt solutions of various concentrations showed that the range of error was considerable, being in the neighborhood of ± 10 per cent.

The heat production in blood and serum was measured by this method. As might be anticipated, it was found that whereas the heat production in various samples of serum was the same, that in blood increased as the cell volume was increased. For example, the ratio $\frac{H \text{ Blood}}{H \text{ Serum}}$ (where H represents heat production per unit of time) was found to increase from 1.48 to 3.13 when the cell volume was increased from 36 per cent to 72 per cent (Table I). At the same cell

volumes McClendon found the ratio $\frac{Z \text{ Blood}}{Z \text{ Serum}}$ at $F = 10^6$ to increase from 1.8 to 3.5 and $\frac{Z \text{ Blood}}{Z \text{ Serum}}$ at $F = 10^3$ to increase from 1.8 to 4.7 (Table I) (where Z represents impedance measured by the Wheatstone bridge). It is evident from these figures that as the cell volume is increased the increase of the ratio $\frac{H \text{ Blood}}{H \text{ Serum}}$ resembles the increase in $\frac{Z \text{ Blood}}{Z \text{ Serum}}$ at $F = 10^6$ rather than at $F = 10^3$. This is especially evident at the higher cell volumes, where there is a really significant difference

TABLE I.

Impedance of Blood and Serum.

Cell vol.	H_s	H_b	$\frac{H_b}{H_s}$ $F = 10^6 \times 1.25$	$\frac{Z_b}{Z_s}$ $F = 10^6$	$\frac{Z_b}{Z_s}$ $F = 10^3$
per cent					
36	2.0	2.95	1.48	1.8	1.8
40	1.98	4.07	2.06	1.9	2.1
46.5	2.0	3.33	1.67	2.2	2.4
70	2.0	6.78	3.36	3.4	4.4
72	1.98	6.19	3.13	3.5	4.7

H = Heat produced by 400 milliamperes in 10 minutes.
Z = Impedance (data from McClendon (6)).

between $\frac{Z \text{ Blood}}{Z \text{ Serum}}$ at $F = 10^6$ and $F = 10^3$. That the ratio $\frac{H \text{ Blood}}{H \text{ Serum}}$ is as an average slightly lower than $\frac{Z \text{ Blood}}{Z \text{ Serum}}$ at $F = 10^6$ is probably of some significance, although the difference is within the limits of experimental error. Should the difference be real, it would indicate that some portion of the impedance of blood cells does not lead to heat production.

Measurements were also made on blood and blood laked with saponin but we have not published these figures, as subsequent observations showed that saponin itself produces a marked increase in conductivity.

II. Relative Impedance and Resistance Measurements on Blood, Laked Blood, and Serum.

A circuit was constructed whereby the relative impedance and resistance of two unknowns could be ascertained simultaneously. This was done to obviate the errors arising from current measurements by means of the hot wire milliammeter and from varying atmospheric conditions.

Four identical cells were constructed, each of 200 cc. capacity, and equipped with brass electrodes, the dimensions of which were 7 cm. × 4 cm. The cells were then placed in a circuit as represented in Fig. 2, so that Cell I was in series with Cell III, and Cell II in series with Cell IV. Cells I and III together were in parallel with Cells II and IV. With the same electrolyte in each of the cells, it is obvious that the amount of current, and therefore the rate of heat production in each, should be the same. To calibrate the cells, all four were filled with 0.05 M NaCl and the heat increment measured after the passage of 1,300 milliamperes for 10 minutes. This was repeated 14 times. A slight but constant discrepancy in the heat production in the respective cells was found. The average figures for the 14 observations were:

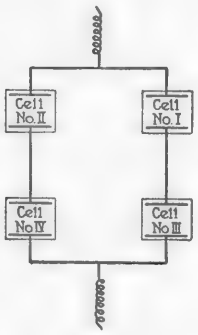

FIG. 2. Circuit used to determine the relative impedance and rate of heat production in two unknowns. Details of the apparatus are described in the text.

Cell I	8.38°C.
Cell II	8.69°C.
Cell III	8.33°C.
Cell IV	8.57°C.

This discrepancy can only be due to some differences in the electrical constants of the cells, or differences in the calibration of the thermometers. Cell I was arbitrarily chosen as the standard and the others corrected on the basis of the above observations. The correction factors used were therefore:

1.000 for Cell I.
0.964 for Cell II.
1.006 for Cell III.
0.977 for Cell IV.

With these corrections the temperature increments in all 4 cells corresponded to within ± 1.7 per cent. These correction factors were used in the experiments about to be described. To correct for heat loss in the cells cooling curves were

constructed with saline and blood. The appropriate correction was applied to the observed temperature increase. All experiments were started with the contents of the cells 0.5–1.5°C. below room temperature. An effort was made to keep the environmental temperature about the cells constant.

The two unknowns, the impedances of which were to be measured, were placed in Cells I and II, respectively, while Cells III and IV were filled with 0.05 M NaCl. The current was then turned on so that the milliammeter registered 1,300, and after 10 minutes the temperature increment in all four cells was measured. (This temperature increment will be referred to as H.) Since the circuit was constructed so that, apart from the contents of the cells, its impedance was very small, we can say that the expression $\frac{Z_1 + Z_3}{Z_2 + Z_4}$ represents the inverse ratio of the currents passing through the two parallel arms of the circuit (where Z_1 represents the impedance of the contents of Cell I, Z_2 the impedance of the contents of Cell II, etc.). The amount of current passing through Cell I is the same as that passing through Cell III, and the amount of current passing through Cell II is the same as that passing through Cell IV. Hence we can write:

$$\frac{Z_1 + Z_3}{Z_2 + Z_4} = \frac{I_2}{I_1} = \frac{I_4}{I_3}$$

Since Cells III and IV contain an electrolyte of the same conductivity, we can write $\frac{I_4}{I_3} = \frac{\sqrt{H_4}}{\sqrt{H_3}}$.
It follows that:

$$\frac{Z_1 + Z_3}{Z_2 + Z_4} = \frac{\sqrt{H_4}}{\sqrt{H_3}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Since Z_3 and Z_4 are identical, the figure $\frac{\sqrt{H_4}}{\sqrt{H_3}}$ will tell us whether $\frac{Z_1}{Z_2}$ is greater or less than unity.

If we assume that the contents of the four cells have a similar dielectric constant, an assumption which seems reasonable since they consist of electrolytes with no great divergence of conductivity, we can obtain from these heat measurements a figure which will represent quantitatively the ratio $\frac{R_1}{R_2}$, where R represents that part of the impedance which leads to heat production. Since the amount of current flowing through Cells I and III is the same and that flowing through II and IV is the same, we can write $\frac{R_1}{R_3} = \frac{H_1}{H_3}$ and $\frac{R_2}{R_4} = \frac{H_2}{H_4}$. It follows that

$\frac{R_1}{R_3} \Big/ \frac{R_2}{R_4} = \frac{H_1}{H_3} \Big/ \frac{H_2}{H_4}$, which can be written $\frac{R_1}{R_3} \times \frac{R_4}{R_2} = \frac{H_1}{H_3} \times \frac{H_4}{H_2}$. Since Cells III and IV both contain 0.05 M NaCl, R_4 and R_3 can be cancelled out so that:

$$\frac{R_1}{R_2} = \frac{H_1}{H_3} \times \frac{H_4}{H_2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The accuracy of this method was checked by a series of observations on various electrolyte solutions, of which the conductivity had been measured by a Wheatstone bridge at a frequency of 10^3. There is no reason to suppose that conductivity measurements by the low frequency Wheatstone bridge should not be applic-

TABLE II.

Saline and Saponin Controls.

Contents of cells			$\frac{R_1}{R_2}$		$\frac{Z_1 + Z_3}{Z_2 + Z_4}$	
I	II	III and IV	$F = 10^6 \times 1.25$	$F = 10^3$	$F = 10^6 \times 1.25$	$F = 10^3$
0.04 M NaCl	0.06 M NaCl	0.05 M NaCl	1.474	1.439	1.171	1.195
			1.429	1.439	1.198	1.195
			1.427	1.439	1.201	1.195
			1.473	1.439	1.211	1.195
0.55 M NaCl	0.05 M NaCl	0.05 M NaCl	0.915	0.916	0.957	0.955
			0.930	0.916	0.971	0.955
0.05 M NaCl	0.05 M NaCl + 1 per cent saponin	0.05 M NaCl	1.317	1.302	1.130	1.132
			1.278	1.302	1.139	1.132

Z = Impedance of contents of cell.
R = Impedance represented by heat production.

able to the diathermy current when simple electrolytes are used. In all the experiments the unknowns were placed in Cells I and II while Cells III and IV were filled with 0.05 M NaCl. With 0.04 M NaCl and 0.06 M NaCl as the unknowns, the average values for $\frac{R_1}{R_2}$ and $\frac{Z_1 + Z_3}{Z_2 + Z_4}$ were 1.451 and 1.195 with the diathermy current, 1.439 and 1.195 with the Wheatstone bridge. With 0.055 M NaCl and 0.050 M NaCl the figures were 0.922 and 0.964 with diathermy and 0.916 and 0.955 with the Wheatstone bridge. With 0.05 M NaCl and 0.05 M NaCl + 1 per cent saponin, the figures were 1.297 and 1.134 with diathermy and 1.302 and 1.132 with the Wheatstone bridge (Table II). These controls clearly show that even slight differences in impedances can be measured by this method with a considerable degree of accuracy.

Similar experiments were carried out with blood cells and laked blood cells, and also with blood cells and serum, as the unknowns. The material used was defibrinated ox blood, obtained fresh from the slaughter house. The serum was separated and the cell volume varied by centrifugalization. The blood cells were laked by freezing and thawing. As in the control experiments, the unknowns were placed in Cells I and II, while Cells III and IV were filled with 0.05 M NaCl. It was found that whereas laking produced no appreciable change in

TABLE III.

Impedance of Blood and Laked Blood.

Hematocrit reading	$F = 10^6 \times 1.25$	$F = 10^3$	$F = 10^6 \times 1.25$
	$\frac{R \text{ Blood}}{R \text{ Laked blood}}$	$\frac{Z \text{ Blood}}{Z \text{ Laked blood}}$	$\frac{Z \text{ Blood} - Z\ 0.05\ \text{M NaCl}}{Z \text{ Laked blood} - Z\ 0.05\ \text{M NaCl}}$
73	0.976	1.31	0.986
	0.985	1.31	0.990
50	0.925		0.959
	0.945		0.969
47	0.987	1.092	1.013
	0.997		1.006
	0.990		1.013
39	0.965		0.969
	0.990		0.996
	0.963		0.975
Average.....	0.973		0.997

Z = Impedance.

R = Impedance represented by heat production.

the impedance of blood, the rate of heat production was increased by about 3 per cent. The average of the 10 observations made was 0.997 for the value of $\frac{Z \text{ Cells} + Z\ 0.05\ \text{M NaCl}}{Z \text{ Cells laked} + Z\ 0.05\ \text{M NaCl}}$ and 0.973 for the value of $\frac{R \text{ Cells}}{R \text{ Cells laked}}$ (Table III). Since presumably all the impedance of laked blood is ohmic in character, this can only mean that about 3 per cent of the impedance of intact cells is inductive, and therefore does not lead to the production of heat.

With blood of 72 per cent cell volume and serum as the unknowns, the ratio $\frac{R\,\text{Blood}}{R\,\text{Serum}}$ was found to average 2.5. McClendon found that at the same cell volume $\frac{Z\,\text{Blood}}{Z\,\text{Serum}}$ was 3.5 at $F = 10^6$ and 4.7 at $F = 10^3$. Thus we again have $\frac{R\,\text{Blood}}{R\,\text{Serum}}$ somewhat lower than $\frac{Z\,\text{Blood}}{Z\,\text{Serum}}$ at the same frequency and very much lower than $\frac{Z\,\text{Blood}}{Z\,\text{Serum}}$ at low frequencies (Table IV). The discrepancy between $\frac{R\,\text{Blood}}{R\,\text{Serum}}$ and $\frac{Z\,\text{Blood}}{Z\,\text{Serum}}$ at

TABLE IV.

Impedance of Blood and Serum.

Cell vol.	$\frac{R\,\text{Blood}}{R\,\text{Serum}}$	$\frac{Z\,\text{Blood}}{Z\,\text{Serum}}$	$\frac{Z\,\text{Blood}^*}{Z\,\text{Serum}}$	$\frac{Z\,\text{Blood}^*}{Z\,\text{Serum}}$
	$F = 10^6 \times 1.25$	$F = 10^6$	$F = 10^6$	$F = 10^6$
per cent				
72	2.60	4.36	3.5	4.7
72	2.42	4.41	3.5	4.7

Z = Impedance.
R = Impedance represented by heat production.
* Data from McClendon (6).

the same frequency confirms the previous observation that part of the impedance of blood cells does not lead to the production of heat. The difference, however, is more than one would expect from the results obtained on whole and laked blood. Some of this difference can no doubt be explained by differences in the technic employed for making hematocrit readings by McClendon and ourselves. However, if we make use of the impedance measurements at $F = 10^3$ (Table IV), as a standard of comparison, a considerable discrepancy still exists $\left(\frac{R\,\text{Blood}}{R\,\text{Serum}} = 2.5 \text{ and } \frac{Z\,\text{Blood}}{Z\,\text{Serum}} = 3.3\right)$.

DISCUSSION.

The results of these experiments seem to show pretty conclusively that, with regard to its passage through biological media, the dia-

thermy current behaves in the same manner as low voltage high frequency currents of the pure sine wave form. With both, the laking of cells produces no change in impedance, and with both, an increase in cellular concentration of blood produces an impedance change which is characteristically lower than that found with low frequency currents. The passage of the diathermy current through the living cell can then be represented by Fig. 1. The cell membrane is a very efficient condenser which transmits the current by its capacitance with little or no dielectric loss and consequently little or no stress and strain on the "cell wall." Cellular massage is a term commonly met with in the literature on diathermy. There is no evidence to show that either this, or the electromechanical vibration which is said to occur with currents of a higher frequency, exists.

From theoretical considerations we should not expect that the addition of a glucoside such as saponin to an electrolyte would produce any appreciable change in the conductivity. To our surprise, however, we found that a very definite increase in conductivity occurred (Table II). This increase appears to be independent of voltage and frequency, so it must be a true conductivity change. No attempts were made to determine the purity of the saponin used, but it was obtained from C. A. F. Kahlbaum of Berlin and classified as purified saponin. Others working on the conductivity of biological media under various conditions seem to be unaware of this phenomenon (6) and we, ourselves, were for some time led astray by it.

SUMMARY.

1. A method is described for measuring the relative impedance of living cells to diathermy currents.

2. The diathermy current penetrates the living cell, and heat production is intracellular as well as extracellular.

3. A small proportion of the impedance of living cells to the diathermy current seems not to lead to the production of heat.

4. Evidence is given that the addition of saponin produces an appreciable increase in the conductivity of an electrolyte. Its use is therefore contraindicated when electrical measurements are being made on biological material.

5. The currents used in diathermy behave as do high frequency

currents of the pure sine wave form in respect to their passage through biological material.

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STUDIES ON BACILLUS TYPHOSUS TOXIC SUBSTANCES.

I. Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.*

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Plates 6 and 7.

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INTRODUCTION.

Brieger (1) initiated the studies on toxic substances derived from *B. typhosus* cultures. Schütze (2) claimed that these substances possessed no antigenic properties. Further work on the nature of the toxic factors was done by Sirotinin (3), and Beumer and Peiper (4). Bitter (5), Chantamesse (6), Besredka (7), Rodet and Lagriffoul (8), Kraus and Stenitzer (9), Arima (10) and others obtained immune sera which were supposed to neutralize the toxic substances derived from *B. typhosus* cultures grown in fluid and solid media. Pfeiffer and Bessau (11) showed that immune sera had no superior neutralizing properties over normal sera and that there was no neutralization according to the law of multiple proportions. The toxic substances of *B. typhosus* have been claimed to be heat resistant by Sirotinin (3), Chantamesse (6) and Besredka (7). According to Kraus and Stenitzer (9) these substances are not influenced by light or by exposure to room temperature. The majority of the authors considered the toxic substances as endotoxins (Pfeiffer (11), Besredka (7) and others). Kraus and Stenitzer (9) and later Arima (10) were of the opinion that the typhoid cultures contain both endotoxins and exotoxins. The symptoms produced by the toxic substances are devoid of any specific features. In acutely poisoned rabbits they consist of convulsions, Cheyne-Stokes respiration, diarrhea, paralysis and, possibly, swelling of Peyer's patches (Arima (10)). Similar symptoms have been demonstrated by injection of anaphylatoxins (Friedberger (12)).

On the basis of the work thus far mentioned, a general belief arose that no true toxins exist in *B. typhosus* cultures. In fact, the classical requirements for demonstration of a true toxin, namely, heat lability, serum neutralization in multiple proportions and specific pathological effect upon animals were not fulfilled. The subject has been revived by Zinsser (13) in recent years. The toxic substances obtained from fluid cultures of various microorganisms, including *B. typhosus*,

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were called by him x-substances. They produced no specific pathological changes when injected into animals. Antigenic properties could not be determined because of fluctuations in the response of the animals and inability to continue immunization over a prolonged period of time. The x-substances were also heat resistant, were more toxic for rabbits than for guinea pigs and their effect upon animals was always accompanied by a short but definite incubation period. Zinsser thought that they could not be dismissed merely as split products and considered them somewhat similar to exotoxins as reported by Kraus and Stenitzer, and by Arima.

The present author has carried out experiments as preliminary to the work here to be reported, which can be summarized as follows:

Whole cultures and filtrates of cultures of *B. typhosus* grown under various conditions presumably favorable to the production of powerful toxins were injected intravenously into rabbits. The symptoms produced by the intravenous injections were similar to those described by previous authors. The susceptible animals became sick shortly after the injection. Before death there was very profuse diarrhea, increased respiration, paralysis and, frequently involuntary muscular contractions. Analysis of the highly inconstant results of titration of the toxic effect in a large group of rabbits showed the impossibility of establishing any relation between the dose of the toxic substances injected and the reaction of the animals. The inconstancy of the effect was most likely due to individual variations in the susceptibility of the animals to the effect of these substances.

The effect of *B. typhosus* culture filtrate upon the skin of normal rabbits was also studied. About 50 per cent of all the normal rabbits tested by skin injections of the filtrate showed no reactions whatsoever. In the remaining rabbits the skin injections produced erythemas. Only a small percentage of the positively reacting animals (12 per cent) gave well pronounced erythemas. A majority of positive rabbits reacted weakly. Moreover, different areas of the skin of the abdomen of the same rabbits presented considerable variations in the intensity of reactions to *B. typhosus* culture filtrate.

As can be seen from this summary, the general response and the skin reactions of rabbits to *B. typhosus* culture filtrate could not be used as criteria for studies on the nature of the toxic factors of these filtrates. Further experiments now to be described demonstrated a phenomenon of local skin reactivity to *B. typhosus* culture filtrates.

Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.

Methods.—The *strains* employed for this work were T_L and T_{240}. They were obtained from the stool of convalescent typhoid fever patients 1 and 4 years ago, respectively. Both strains appeared smooth and were easily agglutinated by *B. typhosus* antisera to a high titer. The floccules were large. The strain T_{240}, however, was inagglutinable during the 1st month of cultivation.

The *toxic* substances were prepared as follows:

200 cc. of tryptic digest broth of initial pH 7.8 were added to 2000 cc. Erlenmeyer flasks to give a large surface area. The entire growth of one 24 hour old agar slant culture of *B. typhosus* was suspended in 10 cc. of salt solution. 10 cc. of the suspension were added to each Erlenmeyer flask. The period of incubation was 6 days. The cultures were then filtered through paper and cotton and finally through Berkefeld V candles. The filtrates were tested for sterility, stored in the refrigerator and used for a period of approximately 2 to 3 weeks following their preparation. No preservative was added.

The entire skin of the abdomen of rabbits was epilated with barium sulfate. The animals were injected not less than 24 hours after shaving if no signs of irritation were seen. For the local injections described in this paper, the needle was inserted subcutaneously and then pushed upwards until the point was just seen through the skin. 4 areas, near the upper and lower right and left corners of the epilated areas, about 2 inches apart from each other, were used for the injections. The amount of fluid injected into each area was usually 0.25 cc.

The type of the reaction to be described in the following part of the paper was termed *hemorrhagic*. The severity was expressed in pluses. The size of the reaction was recorded. Distinction was made between the hemorrhagic reaction and the erythema described further on which varied from light pink to a distinctly red color.

Experimental: Protocol 1.—Upper and lower right and left areas of the skin of the abdomen of Rabbits 14-1 and 14-2 were injected each with 0.5 cc. of T. D. T_L filtrate. 24 hours later Rabbit 14-1 showed no skin reaction, Rabbit 14-2 a slightly pink erythema (2+) in the injected areas. The same morning intravenous injections of 3 cc. per kilo of body weight of the filtrate were given to both rabbits. The skin of the animals was examined every hour afterwards. Approximately, 2 hours after the intravenous injections blue discoloration appeared at the site of previous skin injections in Rabbit 14-1. The skin of Rabbit 14-2 remained unchanged. The discoloration observed in Rabbit 14-1 rapidly increased until the reaction became extremely pronounced in about 4 hours after the intravenous injection. At this time, all 4 areas were dark blue in the center with a deep red zone at the periphery. The skin over the hematomas was glossy and swollen. The size of these areas was considerable, the reaction in the upper right corner measuring 2 × 2 cm., the lower right 3 × 2 cm., the upper left 4 × 2 cm. and the lower left corner 3 × 2½ cm. The reaction described resembled a severe bruise. The animal was killed 5 hours after the intravenous injection and sections of the

skin were made. Rabbit 14-2 showed no reaction in the course of 24 hours following the intravenous injection.

Protocol 2.—4 areas of the skin of the abdomen of Rabbit 7-6 were injected each with 0.25 cc. of T. D. T_{240} filtrate. 20 hours later there were "2+" erythemas at the site of skin injections. 24 hours after the skin injections this rabbit was injected intravenously with the filtrate in a dose of 1 cc. per kilo of body weight. The previously injected skin areas showed discoloration in about 2 hours after the intravenous injection. The reactions again, progressively, increased and 4½ hours after the intravenous injection they were extremely severe. The areas were hemorrhagic. They appeared dark blue, glossy and swollen. The upper right corner reaction measured 3½ × 3 cm., the lower right 2 × 2½ cm., the upper left 2 × 2 cm. and the lower left 1½ × 3 cm. Rabbit 7-6 was reexamined 24 hours later. The areas were then black with a dark red zone at the periphery. The size remained unchanged. It appeared that the reaction reached its maximum size in about 5 hours after the intravenous injection. The healing of the hemorrhagic areas was slow. Sloughs formed in about 48 hours after the intravenous injection. The sloughs were followed by scab formation, their gradual separation and scarring. The complete process of healing took about 8 days.

Histological examinations of the hemorrhagic areas were made.

Sections of these areas from Rabbit 14-1 obtained 5 hours after the intravenous injection can be described briefly, as follows:

The general impression was that of the severest type of hemorrhage and necrosis. The skin was edematous in places. Some of the blood vessels were ruptured. The subcutaneous tissue was engorged with blood. There was also an extensive migration of polymorphonuclear neutrophil leucocytes. There was observed pronounced necrobiosis of these cells located inside and outside the blood vessels. Some of the blood vessels contained small parietal thrombi. While it was clear that the process affected the veins, it remained unsettled whether there was any primary injury to the arteries. Some of the arteries were found normal. Others were almost entirely obliterated. The obliteration, however, was probably secondary to the hemorrhagic infiltration outside the arteries. Occasionally, hyalinization was seen in the blood vessels. The hemorrhage and necrosis extended to the corium of the skin, which was thin and broken in places.

The sections of the hemorrhagic areas of Rabbit 7-6 made 24 hours after the intravenous injection were almost identical with the sections of Rabbit 14-1.

More extensive histological studies of the phenomenon are under progress.

As is seen from these protocols, a phenomenon of local skin reactivity to *B. typhosus* culture filtrate was observed. The reactivity was due to skin injection of the filtrate 24 hours prior to the intravenous injection of the same filtrate. The local response was that of severest hemorrhage and necrosis and was fully developed 4 to 5 hours

after the intravenous injection.[1] This phenomenon was reproduced in many rabbits.

In order to determine the characteristic features of the phenomenon the following points were investigated:

1. Susceptibility of Normal Rabbits to the Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrates.

In the course of the work it was observed that certain animals did not respond to the above described treatment. Statistical data were accumulated in order to determine the percentage of normal rabbits which were susceptible to the phenomenon of local reactivity to *B. typhosus* culture filtrates.

In this group of experiments filtrates of Strain T_L or T_{240} cultures in tryptic digest broth were employed. Each area of the skin was injected with 0.25 cc. of undiluted or diluted 1:2 culture filtrate. The dose for intravenous injection varied from 1 to 3 cc. per kilo of body weight. The interval between the skin and intravenous injections was from 20 to 24 hours. Some of the animals died shortly after the intravenous injection (1 to 3 hours) or in the course of the following 48 hours. A considerable percentage of rabbits survived 48 hours. Although in many cases it was already possible to make readings of the reactions 2 hours after the intravenous injection the rabbits which died earlier than 3 hours after the intravenous injection were not taken into consideration in this part of the work.

In the animals which were considered resistant to the phenomenon there was no local hemorrhagic necrosis following the intravenous injection. When, prior to the intravenous injection, there was erythema at the site of skin injections, it became more pronounced 4 to 5 hours after the intravenous injection. Frequently swellings appeared. 24 hours later the skin appeared normal again.

The positively reacting animals showed very severe hemorrhagic necrosis at the site of preliminary skin injections. No mild reactions which would constitute an intermediate group between the negative and positive animals were obtained under the conditions of this part of the work. Of the 212 animals tested in this manner, there were 45 negatively reacting rabbits and 167 rabbits which showed severe reactions (approximately, 78 to 79 per cent positive animals).

[1] The factors which induced the local skin reactivity are termed *"skin preparatory factors"* and those involved in production of local hemorrhagic reactions following the intravenous injection *"reacting factors."*

TABLE I.

The Uniformity of the Hemorrhagic Reaction in Various Areas of the Skin of the Abdomen.

No. of rabbits	Dose of each skin injection	Dose of intravenous injection per kilo of body weight	Filtrates used for skin and intravenous injections	Size, intensity and type of reaction in various areas of the skin of the abdomen			
				The upper right area of the abdomen	The lower right area of the abdomen	The upper left area of the abdomen	The lower left area of the abdomen
	cc.	*cc.*					
I	0.5	2.8	$T.D.T_L$	Hem. 4+ 2 × 2½ cm.	Hem. 4+ 1½ × 2 cm.	Hem. 4+ 3 × 2 cm.	Hem. 4+ 3 × 2 cm.
II	"	2	$T.D.T_{240}$	–	–	–	–
III	"	3	$T.D.T_L$	Hem. 4+ 2 × 3 cm.	Hem. 4+ 3 × 2 cm.	Hem. 4+ 4 × 2 cm.	Hem. 4+ 3 × 2½ cm.
IV	"	"	"	–	–	–	–
V	0.25	0.5	"	Hem. 4+ 2 × 1½ cm.	Hem. 4+ 2 × 2 cm.	Hem. 4+ 2 × 2 cm.	Hem. 4+ 3 × 1 cm.
VI*	"	3	"	–	–	–	–
VII	"	"	"	Hem. 4+ 2 × 1 cm.	Hem. 4+ 1½ × 2 cm.	Hem. 4+ 3 × 2 cm.	Hem. 4+ 2 × 2 cm.
VIII	"	0.2	$T.D.T_{240}$	Hem. 4+ 2 × 2 cm.	Hem. 4+ 3 × 1 cm.	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1½ × 1½ cm.
IX	"	3	$T.D.T_L$	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1 × 1 cm.	Hem. 4+ 2 × 3 cm.	Hem. 4+ 3 × 1 cm.
X	"	"	"	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1 × 2 cm.	Hem. 4+ 4 × 4 cm.	Hem. 4+ 2½ × 2 cm.
XI	"	"	"	Hem. 4+ 1 × 2 cm.	Hem. 4+ 1 × 1 cm.	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1½ × 2 cm.
XII**	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XIII	"	"	"	Hem. 4+ 1½ × 1½ cm.	Hem. 4+ 1½ × 2 cm.	Hem. 4+ 2½ × ½ cm.	Hem. 4+ 1½ × 2 cm.

XIV	0.5	0.5	"	−	−	−	−
XV	"	0.8	"	Hem. 4+ $3\frac{1}{2} \times 3$ cm.	Hem. 4+ 2×2 cm.	Hem. 4+ 2×2 cm.	Hem. 4+ $1\frac{1}{2} \times 3$ cm.
XVI***	0.25	2	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XVII*	"	"	"	−	−	−	−
XVIII***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XIX	"	"	"	Hem. 4+ 1×1 cm.	Hem. 4+ 2×1 cm.	Hem. 4+ $1\frac{1}{2} \times 2$ cm.	Hem. 4+ $1\frac{1}{2} \times 1\frac{1}{2}$ cm.
XX***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XXI***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XXII****	"	"	"	.....	.....	.....	.....
XXIII****	"	"	"	.....	.....	.....	.....
XXIV****	"	"	"	.....	.....	.....	.....

....., not protocolled.

−, no reaction.

Hem., hemorrhagic.

4+, very severe.

* Died in ½ hour after intravenous injection. No reaction was seen yet.

** Confluent reactions.

*** Not measured. Approximately 2×2 cm. in size each.

**** Died in 1 to 2 hours after intravenous injection. Beginning of reaction.

2. The Uniformity of the Hemorrhagic Reaction in Various Areas of the Skin of the Abdomen.

Experiments were carried out in order to determine whether there was a uniform response of different areas of the skin of the abdomen to the intravenous injection of *B. typhosus* culture filtrates.

Protocol 1.—Rabbit 43-4 received injections of T. D. T_x filtrate into 5 areas, namely, upper and lower right and left corners and the center of the skin of the abdomen. 0.1 cc. was injected into each area. The next day there was a slight erythema (1+) in each injected area. 24 hours after the skin injections the rabbit received 3 cc. per kilo of body weight of the same filtrate intravenously. 4 hours later the previously injected skin areas were severely hemorrhagic. There was no appreciable difference in the *severity* of the reaction in different areas. The upper right area measured $1\frac{1}{2} \times 1$ cm., the other areas were 1×1 cm. in size. 24 hours following the intravenous injection no change in the size and type of reaction was observed.

As is seen from this experiment, 5 different areas of the skin of the abdomen responded to intravenous injection of the filtrate with an equal degree of severity and showed no appreciable difference in size of the reactions.

In view of the importance attached to these findings it was deemed necessary to extend the work to a large group of animals, in which the doses for both skin and intravenous injections varied to a certain extent.

Protocol 2.—A group of 24 rabbits was used. The conditions of the experiments and the results are summarized in Table I. It will be noted from this table that the positively reacting animals showed uniformly extremely severe hemorrhagic reactions in prepared skin areas following intravenous injections of *B. typhosus* culture filtrates in various doses. The only variations observed were in the size of the reaction. The smallest reaction was 1×1 cm. and the largest 4×4 cm. The size of the reaction did not definitely depend on the amount of filtrate used for the skin preparation. Injection of 0.25 cc. into the skin, frequently led to reactions as large and larger than the injection of 0.5 cc. of the filtrates. For the same animals variations in the size of different areas lay between 1 and 3 cm. in diameter, as is seen from Animal X, in which the widest variations were obtained. Ordinarily, the difference was 1 to 2 cm. Occasionally confluent reactions were obtained. No well marked relationship between the intravenous dose and the size of the reaction of the skin was observed under the conditions of these experiments.

3. *Titration of the Skin Preparatory Factors of B. typhosus Culture Filtrates.*

Protocol 1.—In order to titrate the skin preparatory factors of *B. typhosus* culture filtrates various dilutions were injected into the skin of the abdomen. 24 hours later the undiluted filtrate was injected intravenously in a dose of 3 cc. per kilo of weight. Readings of the reactions were made 5 and 24 hours after the intravenous injections. In this group of experiments T. D. T_L filtrate was employed.

The results obtained on 12 rabbits susceptible to the phenomenon can be summarized as follows:

The filtrate diluted up to 1:4 was consistently able to induce the local skin reactivity. Reactions following the intravenous injections were severely hemorrhagic, necrotic and varied in size from 1 × 1 cm. to 3 × 2 cm.

There were observed fluctuations in the preparatory effect of higher dilutions. Filtrate dilutions from 1:8 to 1:64 were able to induce local skin reactivity in some animals. The severity of the reactions following the intravenous injections varied from slight blue (1+) to deep blue with an angry red zone at the periphery (4+). It would be of interest to determine whether the fluctuations observed would serve as an indicator of the degree of susceptibility of the rabbits to the phenomenon of local skin reactivity to *B. typhosus* culture filtrates.

Dilutions higher than 1:64 failed to induce the local skin reactivity to *B. typhosus* culture filtrate.

Similar results were obtained with a mixture of equal parts of filtrates derived from different strains (Mt. Sinai, 240, 215_A, 215_B, T_L).

4. *Relation of Intensity of Erythema Following Preparatory Skin Injection to the Local Hemorrhagic Reaction Produced by Intravenous Injection of B. typhosus Filtrate.*

The purpose of the observations reported here was to determine whether the skin reaction following the preparatory skin injection had any influence on the size and severity of the local hemorrhagic response produced by intravenous injection of *B. typhosus* culture filtrate.

Rabbits were injected into 4 areas of the skin of the abdomen with 0.25 cc. of T. D. T_L filtrate. The intensity of the erythema obtained 20 to 24 hours later

TABLE II.

The Relation of the Erythema Following Skin Injection of B. typhosus Filtrate to the Hemorrhagic Reaction Following Intravenous Injection of the Same Filtrate.

No. of rabbits	*Upper right corner*		*Lower right corner*		*Upper left corner*		*Lower left corner*	
	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection
XXVI	Negative	3.5 × 4.5 cm. Hem. 4+	Negative	5 × 3 cm. Hem. 4+	–	–	–	–
XXVII	2+	2 × 2 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+	2+	3 × 1 cm. Hem. 4+	2+	1½ × 1 cm. Hem. 4+
XXVIII	Doubtful	1½ × 1 cm. Hem. 4+	"	1 × 1 cm. Hem. 4+	Doubtful	3 × 2 cm. Hem. 4+	Doubtful	2 × 2 cm. Hem. 4+
XXIX	3+	1 × 1½ cm. Hem. 4+	3+	1 × 1½ cm. Hem. 4+	3+	1 × 1 cm. Hem. 4+	–	–
XXX	3+	1 × 1½ cm. Hem. 4+	2+	1 × 1 cm. Hem. 4+	1+	1 × 1½ cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+
XXXI	2+	2 × 3½ cm. Hem. 4+	2+	4 × 1½ cm. Hem. 4+	2+	4 × 3 cm. Hem. 4+	"	1½ × 2 cm. Hem. 4+
XXXII	Negative	2 × 2 cm. Hem. 4+	Negative	2 × 2 cm. Hem. 4+	Negative	2½ × 1½ cm. Hem. 4+	–	–
XXXIII	"	1½ × 1 cm. Hem. 4+	–	–	1+	1 × 1 cm. Hem. 4+	Negative	1½ × 1½ cm. Hem. 4+
XXXIV	"	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1 cm. Hem. 4+	–	–	"	1½ × 2 cm. Hem. 4+
XXXV	2+	1× 2 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+	4+	1½ × 1 cm. Hem. 4+	2+	2 × 2 cm. Hem. 4+

XXXVI	3+	1½ × 1½ cm. Hem. 4+	3+	1½ × 2 cm. Hem. 4+	Doubtful	1½ × 1 cm. Hem. 4+	Doubtful	1½ × 2 cm. Hem. 4+
XXXVII	1+	1½ × 1 cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+	2+	1 × 2 cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+
XXXVIII	Negative	3 × 3 cm. Hem. 4+	"	3 × 3 cm. Hem. 4+	Negative	2 × 2 cm. Hem. 4+	"	1 × 1 cm. Hem. 4+
XXXIX	2+	2½ × 2 cm. Hem. 4+	"	3 × 1½ cm. Hem. 4+	2+	1 × 3½ cm. Hem. 4+	"	1 × 1 cm. Hem. 4+
XL	1+	1 × 2 cm. Hem. 4+	–	–	2+	1 × 1 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+
XLI	Negative	2½ × 2 cm. Hem. 4+	–	–	Negative	2 × 2 cm. Hem. 4+	"	2 × 2 cm. Hem. 4+
XLII	"	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1½ cm. Hem. 4+	3+	1½ × 2 cm. Hem. 4+	"	2 × 1 cm. Hem. 4+
XLIII	"	1 × 1 cm. Hem. 4+	2+	1 × 1 cm. Hem. 4+	–	–	–	–
XLIV	3+	1½ × 1½ cm. Hem. 4+	2+	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1 cm. Hem. 4+	–	–
XLV	Negative	3 × 2½ cm. Hem. 4+	Negative	2 × 1 cm. Hem. 4+	–	–	Negative	2½ × 2½ cm. Hem. 4+
XLVI	1+	1½ × ½ cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+
XLVII	2+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	–	–	–	–
XLVIII	4+	2 × 2 cm. Hem. 4+	2+	2 × 2 cm. Hem. 4+	4+	2 × 2 cm. Hem. 4+	–	–

–, not tested.

was recorded. 24 hours after the preparatory injections the same filtrate was injected intravenously into these animals. The dose varied from 2 to 3 cc. per kilo of body weight. Readings recorded here were made 24 hours after the intravenous injections.

Some of the animals died 1 to 2 hours after the intravenous injection. The readings of these animals were not included in the protocols.

In Table II, animals susceptible to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate are recorded. It is seen from this table that a considerable number of areas which did not react to the local injection responded severely locally to the intravenous injection of the filtrate. In addition, areas which had erythemas of various intensity showed an approximately equally active response to the intravenous injections.

In this experiment there were also 5 rabbits which were not included in Table II. 2 animals showed no erythema following the preparatory skin injections. 3 rabbits had erythema of various intensity from 1+ to 4+. All these animals were entirely resistant to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate.

As is seen from the observations reported above, there was no relationship between the intensity of the erythema following the preparatory skin injections and the size and intensity of the local hemorrhagic response to intravenous injections of *B. typhosus* culture filtrate. Evidently, the local trauma produced by the preparatory skin injections was not responsible for the skin localization of the toxic factors introduced by the intravenous route. This finding was confirmed by the observation that some animals resistant to the phenomenon of local hemorrhagic response showed erythemas of various intensity after the preparatory skin injections.

5. Attempts to Produce Local Skin Reactivity to B. typhosus Culture Filtrate by Skin Injections of Various Substances.

A series of experiments was performed in order to determine whether the local skin reactivity to *B. typhosus* culture filtrate could be produced by skin injections of various substances.

A: The purpose of the following experiment was to determine whether uninoculated culture medium by itself was able to induce the local skin reactivity to *B. typhosus* culture filtrates.

Protocol 1.—5 rabbits were used for this experiment. The upper and lower right and upper left areas of the skin of the abdomen were injected each with 0.5 cc. of sterile tryptic digest broth. The lower left areas were injected with 0.5 cc. of *B. typhosus* culture filtrate. 24 hours later these rabbits were injected intravenously each with *B. typhosus* culture filtrate. The dose was 3 cc. per kilo of weight. 1½ hours after the intravenous injections 1 rabbit died. No readings were made. 5 hours after the intravenous injections the remaining rabbits showed no reactions in the upper and lower right and upper left areas. The lower left areas of the skin of these rabbits showed very severe hemorrhagic necrosis. The size of the reactions varied from 2½ × 2½ cm. to 3½ × 4 cm.

As is seen from this experiment, sterile tryptic digest broth failed to produce local skin reactivity to *B. typhosus* culture filtrate.

B: Experiments were performed in order to determine whether culture filtrates of various strains of streptococci were able to induce local skin reactivity to *B. typhosus* culture filtrate:

Protocol 1.—In addition to the *B. typhosus* culture filtrate, 2 strains of green producing streptococci (530 and 941) were employed in this experiment. Both strains of streptococcus were isolated from the blood of cases of subacute bacterial endocarditis. The culture filtrates were made in a manner identical with that employed for the preparation of *B. typhosus* culture filtrates.

Rabbits 14-3 and 14-4 received into the upper and lower right areas of the skin injections of streptococcus filtrates 530 and 941, respectively. The upper and lower left areas were injected with T. D. T_L filtrate. 24 hours after the skin injections no reactions were seen. The rabbits were immediately injected intravenously with T. D. T_L filtrate in a dose of 3 cc. per kilo of weight. 5 hours after the intravenous injections no reactions were seen in the upper and lower right areas. The upper and lower left areas, however, showed very severe hemorrhagic reactions. The size of the reactions varied from 1 × 1 cm. to 3 × 2 cm.

Protocol 2.—For this experiment filtrates of 2 strains of *pyogenes Streptococcus hæmolyticus* were prepared in the same manner as *B. typhosus* culture filtrates. In addition toxin of *Streptococcus erysipelatis*, kindly sent to me by Dr. K. Birkhaug under the name E_1—E_6, was employed. A group of 10 rabbits was prepared by skin injections of these filtrates into the upper and lower right and upper left areas. The lower left areas were injected with T. D. T_L filtrate. 24 hours later the rabbits were each injected intravenously with T. D. T_L filtrate. The dose was 3 cc. per kilo of weight. In 1 to 2 hours after the intravenous injections 3 rabbits died. Readings were made 5 hours after the intravenous injections. In 6 rabbits the lower left areas showed very severe hemorrhagic reactions (4+) which varied in size from 1 × 1 cm. to 3 × 2 cm. The other prepared areas were entirely negative. 1 rabbit reacted negatively in all the 4 prepared areas.

It is evident from the experiments of Protocols 1 and 2 that no local skin reactivity to *B. typhosus* culture filtrate was induced by skin injections of 5 different streptococcus culture filtrates. The susceptibility of the rabbits employed to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate was controlled.

C: In this group of experiments the skin of rabbits was prepared by injections of turpentine and 24 hours later *B. typhosus* culture filtrate was injected intravenously.

Protocol 1.—Rabbits 49-4, 49-5, 49-6 were injected with turpentine into 4 areas of the skin of the abdomen. The upper and lower right areas were injected with dilution 1:10 and the upper and lower left areas with dilution 1:5. 24 hours later there were no abscesses in turpentine injected areas, but they were distinctly red. The intravenous injections of potent *B. typhosus* culture filtrate (T. D. T_L) in a dose of 3 cc. per kilo of weight produced no reactions in the prepared areas.

Protocol 2.—The upper and lower right and left areas of the skin of the abdomen of Rabbit 6-3 and 6-9 were injected with 0.2 cc. of undiluted turpentine and turpentine diluted 1:10, 1:20 and 1:40, respectively. 24 hours later the upper right corners of the skin of the abdomen of both animals showed well formed abscesses, the remaining areas showed reddening, but no pus was seen. 24 hours after the skin injections, T. D. T_{240} filtrate, which proved to be potent on the day of the experiment, was injected intravenously into these animals. The dose was 3 cc. per kilo of weight. No local skin reactions followed the intravenous injections.

From the observations reported in this part of the work it was concluded that non-specific irritating substances such as sterile tryptic digest broth and turpentine, and culture filtrates of certain microorganisms biologically unrelated to *B. typhosus* could not substitute the skin preparatory factors of *B. typhosus* culture filtrate. Additional studies are under way in order to determine whether the local skin reactivity to *B. typhosus* culture filtrate can be produced by microorganisms biologically related to *B. typhosus*.

6. The Effect of Heat upon the B. typhosus Skin Preparatory Factors.

To determine the effect of heat upon the skin preparatory factors a number of experiments was made:

Protocol 1.—Rabbits 28-0, 28-1 and 28-2 were injected into the upper and lower right and left areas of the skin of the abdomen respectively with unheated T. D. T_L diluted 1:2, with the same dilutions of T. D. T_L heated to 60°C. for 1 hour with 1:2 T. D. T_L heated in the Arnold steam sterilizer for 1 hour and with 1:2 T. D.

T_L heated in the autoclave for 45 minutes. 24 hours after the skin injections Rabbits 28-0 and 28-1 showed no reactions and Rabbit 28-2 had a diffuse redness extending over the entire skin of the abdomen (2+). 24 hours after the skin injections these rabbits received intravenously unheated T. D. T_L filtrate in a dose of 3 cc. per kilo of body weight. 5 hours after the intravenous injections the upper and lower right and upper left areas of all 3 rabbits showed severe hemorrhagic reactions which varied in size. The lower left areas were entirely negative. Rabbit 28-2 died 6 hours after the intravenous injection. Rabbits 28-0 and 28-1 showed no change in reactions 24 hours after the intravenous injection.

These experiments were repeated on 10 more rabbits with identical results. The slight variations in the size of severe hemorrhagic reactions could be disregarded.

As is seen from these observations, the skin preparatory factors of this strain of *B. typhosus* culture filtrate could be inactivated by heating in the autoclave for 45 minutes. Lower temperatures had no appreciable effect on the ability of the filtrate to induce the local skin reactivity.

Protocol 2.—Similar experiments were performed with a filtrate derived from a culture of a different strain of *B. typhosus* (T_{240}). The culture was made in tryptic digest broth in the usual manner. Rabbits 26-5 and 26-6 were injected into the upper and lower right and left areas of the skin of the abdomen respectively with unheated filtrate T_{240}, with T_{240} filtrate heated to 60°C. for 1 hour, with T_{240} filtrate heated in the Arnold steam sterilizer for 1 hour and the T_{240} filtrate heated in the autoclave for 45 minutes. 24 hours later the rabbits were injected intravenously with the unheated filtrate T. D. T_{240}. The dose was 2 cc. per kilo of body weight. 4½ hours after the intravenous injections all the 4 prepared areas showed extremely severe hemorrhagic reactions which varied in size from 2 × 2 cm. to 4 × 3 cm.

Further attempts were made to inactivate the skin preparatory factors of T. D. T_{240} filtrates by autoclaving:

The filtrates were first diluted 1:2 and then autoclaved for 45 minutes. In other experiments the autoclaving of diluted and undiluted T. D. T_{240} filtrate was prolonged to 1 hour. In all the experiments the skin preparatory factors remained unaffected by the process.

Protocol 3.—A mixture of equal amounts of filtrates derived from cultures of 4 different strains of *B. typhosus* (Mt. Sinai, L, 215_A and 215_B) was employed for these experiments. 6 rabbits were used. The experiment was performed in a manner identical with that described in Protocol 1. 1 rabbit died 1 hour after the injection. No reading was made. 4 rabbits showed very severe hemorrhagic

reactions in the upper and lower right and upper left areas. The lower left areas were entirely negative. 1 rabbit was resistant to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate. The same readings were obtained 24 hours after the intravenous injection.

As is seen from the experiments of this part of the work, the skin preparatory factors of *B. typhosus* possessed considerable resistance to heating. Filtrates derived from certain strains lost these factors when autoclaved for 45 minutes. There was encountered, however, a filtrate of one strain (T_{240}) which resisted autoclaving for 1 hour.

7. *The Effect of Different Hydrogen Ion Concentrations upon the B. typhosus Skin Preparatory Factors.*

It was desirable to determine the effect of acid and alkali upon the skin preparatory factors of *B. typhosus* and, incidentally, to determine whether there is a difference in the heat resistance of filtrates adjusted to various pH.

The experiments were performed as follows:

The *B. typhosus* culture filtrate T. D. T_L was adjusted under sterile precautions to pH 9.0, 8.6, 7.6, 7.0, 6.6, 5.4 and 4.0.[2] The final dilution of the filtrates of various pH was 1:2. The filtrates of pH 9.0 and 4.0 were used on the day of adjustment; those of pH 8.6 to 6.0 were injected 25 hours after the adjustments. Before use, given amounts of filtrates of various pH were heated for 1 hour at 60°C., in the Arnold sterilizer and in the autoclave. A group of 4 rabbits was employed for experiments with heated and non-heated filtrates of each pH. The upper right areas of the skin of the abdomen were injected with the unheated filtrates. Filtrates heated for 1 hour at 60°C., filtrates heated for 1 hour in the Arnold sterilizer and filtrates heated for 1 hour in the autoclave were injected into the lower right and upper and lower left areas, respectively. The center of the skin of the abdomen was injected with non-adjusted and unheated T. D. T_L filtrate diluted 1:2.[3] 24 hours after the preparatory skin injections the rabbits received intravenously the unheated and non-adjusted T. D. T_L filtrate. The dose was 3 cc. per kilo of weight. The susceptible animals reacted severely 5 hours after the intravenous injections. Reactions were obtained in upper and lower right and upper left areas. The lower left areas were entirely negative. The same readings were made 24 hours after the intravenous injection.

[2] No buffers were used for adjustments.

[3] The pH of the filtrate was 7.9 to 8.0.

As is seen from these experiments, there was no inactivation of the skin preparatory factors at a hydrogen ion concentration range of from 9.0 to 4.0 and there was no change in the heat resistance at the various pH. The factors derived from the strain used (T. D. T_L) were invariably inactivated by autoclaving for 1 hour.

8. Incubation Period in Preparation of the Skin to the Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.

In the experiments thus far reported there was allowed an interval of 24 hours between the skin preparatory injections and the intravenous injection of *B. typhosus* culture filtrate. The following experiments were made in order to determine the optimum interval between the injections necessary to elicit the reactivity:

Protocol 1.—6 rabbits (Nos. 48-2 to 48-6) were injected into 4 areas of the skin with T. D. T_L filtrate. Rabbits 48-2, 48-3 and 48-4 received intravenous injections of the filtrate 2 hours after the skin injections. The dose was 3 cc. per kilo of weight. No reactions were seen in these rabbits in the course of the following 48 hours.

Rabbits 48-5, 48-6 and 48-7 were injected intravenously with 3 cc. per kilo of body weight 48 hours after the skin injections. The skin remained unchanged for 48 hours later.

No definite conclusions could be drawn from this experiment, since as shown on page 251 a certain percentage of animals was spontaneously resistant to the phenomenon of local reactivity to *B. typhosus* filtrate. In order to eliminate this objection, the following experiment was performed:

Protocol 2.—The upper right areas of the skin of the abdomen of Rabbits 1-7, 1-8, 2-0, 2-6, 2-7 and 43-9 were injected with 0.25 cc. of T. D. T_L filtrate. 24, 48 and 55 hours later 0.25 cc. of the same filtrate was injected into the lower right, upper left and lower left areas of skin of the abdomen respectively. 56 hours after the first skin injections filtrate T. D. T_L was injected intravenously into these rabbits. The dose was 3 cc. per kilo of body weight. The morning following the intravenous injections namely 15 hours after the intravenous injections the following results were obtained: Rabbit 1-7 was found dead and no reading was possible. Rabbits 1-8, 2-0, 2-6, 2-7 and 43-9 showed no reactions in the upper right and lower left areas. Both lower right and upper left areas showed very pronounced hemorrhagic reactions which varied in size from 1 × 1 to 2 × 2 cm.

From these experiments the following could be concluded: For the reproduction of the described phenomenon a definite interval of time was required between the preparatory skin injection and the intravenous injection of the filtrate.

No definite data were available as yet in reference to the exact number of hours required to allow for the preparation of the skin. It could only be stated that 2 hours were insufficient and that the state of reactivity did not last longer than 32 hours. The phenomenon could be invariably reproduced if an interval of 24 hours was allowed between the skin and intravenous injections.

In Protocol 2 it will be noticed that skin reactivity appeared 8 hours after the skin preparatory injection. In these animals, however, the reading of the skin reaction was made 15 hours following the intravenous injections, instead of after the customary 5 hours. The significance of this observation will be considered in a subsequent report.

9. Local Reaction to Repeated Skin Injections of B. typhosus Culture Filtrates.

In the work described up to this point severe hemorrhagic and necrotic local reactions were obtained by skin-intravenous injections. Although no attempts were made as yet to study the mechanism of the phenomenon, it was of interest to determine whether the intravenous route was essential for the reaction. The following protocol illustrates these attempts:

Protocol 1.—Rabbits 16-0, 16-1 and 16-2 received each 4 skin injections of T. D. T_L filtrate into the usual areas. 0.5 cc. was injected into each area. No reactions were seen in these rabbits 24 hours after the injections. The same areas were then reinjected with the filtrate. The amount of filtrate was again 0.5 cc. for each area. The readings were, then, made every hour for the following 6 hours and again 20 hours later. About 1 to 2 hours after the reinjections there appeared reddening and swellings in the 4 areas. The reddening became very pronounced towards the end of 5 hours (4+ erythema). Rabbit 16-1 was killed and sections of the inflamed skin were made. The following morning Rabbits 16-0 and 16-2 showed reactions of the same type as the day before. They were then given intravenous injections of T. D. T_L filtrate in a dose of 3 cc. per kilo of body weight. 4 hours after the intravenous injections both rabbits showed very extensive hemorrhagic necrotic reactions which varied from 2×3 to 3×4 cm. in size.

The skin section of Rabbit 16-1 showed inflammatory changes. There was an

infiltration of polymorphonuclear neutrophil leucocytes. The leucocytes did not appear necrobiotic. There was no rupture of the blood vessels. No thrombi were found. Some dilatation of the blood vessels was seen in places. The experiment was repeated on 8 more animals with identical results.

As is seen from this experiment repeated skin injections of the filtrate with a 24 hour interval between the injections did not result in the hemorrhagic and necrotic type of reaction described in this paper. The type of the reaction produced by repeated skin injections consisted of a pronounced cellular infiltration, but in contrast to the phenomenon under consideration, there was no breaking up of the cells and no severe damage to the blood vessels. Moreover, the susceptibility of the animals to the hemorrhagic type of reactions was demonstrated, for, 24 hours after the second skin injections, intravenous injections of the *B. typhosus* filtrate were given and there developed severe hemorrhagic and necrotic reactions at the site of previous skin injections. It seems, therefore, that the preparatory skin injections have to be followed by injection of the filtrate through the intravenous route for the reproduction of the phenomenon described.

Further work on the mechanism of the reaction is under progress. It is intended also to amplify the experiments on repeated skin injections with toxic filtrates of different strength.

CONCLUSIONS AND SUMMARY.

A phenomenon of local skin reactivity to *B. typhosus* culture filtrates is described in this report. The reactivity was induced by skin injections of the filtrate followed 24 hours later by an intravenous injection of the same filtrate. The local response consisted of severe hemorrhagic necrosis and was fully developed 4 to 5 hours after the second injection.

About 78 to 79 per cent of the rabbits employed were susceptible to this phenomenon.

Different areas of the skin of the abdomen, when similarly treated, responded with equal severity to the intravenous injection. There were variations in the size of different areas in the same animals.

The intensity and size of the local hemorrhagic reactions were not related to the intensity of the erythema produced by the preparatory skin injections. Following intravenous injection very severe hemor-

rhagic reactions were obtained, in those areas which reacted negatively in this respect to the preparatory skin injections. Evidently, the local trauma produced by the preparatory skin injections was not responsible for the localization of the toxic factors introduced by the intravenous route.

It was necessary to allow a short interval of time between the skin preparatory injections and the intravenous injection, for the reproduction of the phenomenon. An incubation period of 2 hours was insufficient. An interval of 24 hours was invariably sufficient. The ability to react disappeared in 48 hours after the preliminary skin injections.

Repeated direct injections of the filtrate into the same areas of the skin, with an interval of 24 hours between the injections, did not result in reactions similar to the above described hemorrhagic necrosis. The second skin injection was followed by reddening, some swelling and a local accumulation of polymorphonuclear neutrophil leucocytes which showed no signs of necrobiosis. There was no rupture of blood vessels. Skin injections followed after a suitable interval by intravenous injection, were necessary for the reproduction of the severe local hemorrhagic response.

Skin reactivity to *B. typhosus* culture filtrate injected intravenously was not induced by turpentine in various dilutions, sterile tryptic digest broth, culture filtrates of 4 strains of streptococci or by the *Streptococcus erysipelatis* toxin.

It was possible to titrate the skin preparatory factors. Dilutions of the filtrate up to 1:64 were able to induce the local skin reactivity. But, whereas dilutions up to 1:4 invariably prepared the skin so that very severe hemorrhagic reactions followed the second injection in susceptible animals, dilutions from 1:8 to 1:64 were uncertain and their preparatory effect varied in different animals.

The skin preparatory factors showed considerable heat resistance. The heat resistance varied with the strains employed. One strain produced factors totally resistant to heating in the autoclave for 1 hour. However, there was definite and unquestionable inactivation of these factors as derived from other strains when the filtrate was diluted 1:2 and heated in the autoclave.

Various hydrogen ion concentrations in the range from 9.0 to 4.0 had

no effect upon the skin preparatory factors. Heat resistance was not modified by the various pH within this range.

The mechanism of the phenomenon described has not been fully studied as yet. An experimental comparison of it with the manifestations of bacterial allergy of the skin is necessary. There are certain features, however, which *considered together*, distinguish this phenomenon from the known phenomena of bacterial hypersusceptibility. These features are: local reactivity; the short incubation period necessary to induce the local reactivity; the short duration of the state of reactivity; the ability to induce local reactivity by a single skin injection; the severity of the reaction; and the necessity to make the second injection of the toxic agent by the intravenous route.

Studies on the relation of specific antisera to the phenomenon described are under way.

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EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. 5 areas of the skin were injected each with 0.3 cc. of T. D. T_L filtrate. 24 hours later the rabbit received intravenous injection of the same filtrate. The dose was 2.5 cc. per kilo of body weight. Appearance of reaction at the site of prior skin injections 5 hours after the intravenous injection.

FIG. 2. This rabbit was treated in a manner identical with that represented by Fig. 1. In this rabbit there was a confluent reaction extending from the upper right and lower right areas to the center of the skin of the abdomen.

PLATE 7.

FIG. 3. Hematoxylin-eosin. × 230. Section of skin from Rabbit 14-1. Microscopic appearance of hemorrhagic reaction 5 hours after intravenous injection of T. D. T_L. Note necrobiosis of white blood cells.

FIG. 4. Hematoxylin-eosin. × 270. Section of skin from Rabbit 16-1. Microscopic appearance of reaction produced by repeated skin injections of T. D. T_L. No necrobiosis of white blood cells.

FIG. 1.

FIG. 2.

(Shwartzman: *B. typhosus* toxic substances. I.)

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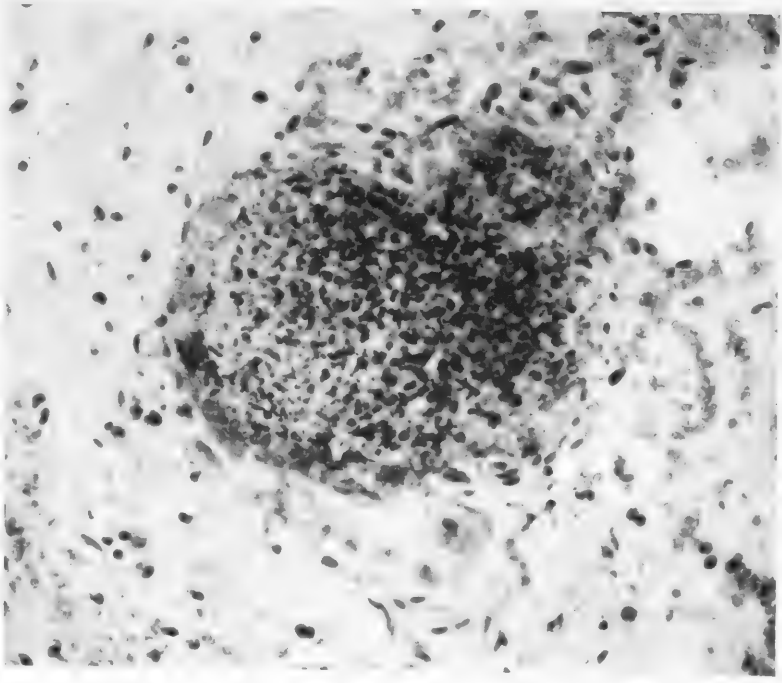

FIG. 3.

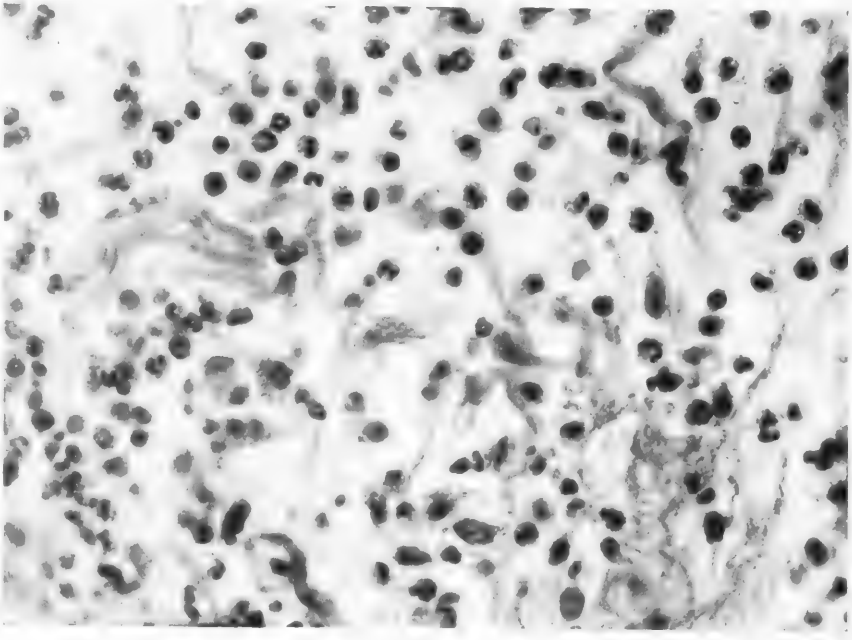

FIG. 4.

(Shwartzman: *B. typhosus* toxic substances. I.)

STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

IX. Evidence of Hydrolysis of Bacterial Protein during Lysis.

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The disappearance of bacteria and the coincident accumulation of an agent capable of bringing about this disappearance in serial subcultures are the characteristics which originally drew the attention of Twort to the phenomenon which later became known under the name of bacteriophagy, or the phenomenon of d'Hérelle. From the outset, Twort saw three distinct possibilities in interpreting his observations: He thought that the disappearance of the bacteria might be due to their passage into a subvisible state as a stage in a peculiar life cycle initiated under the conditions of the experiment. He saw as an alternate possibility that the disappearance of bacteria was due to rapid autolysis of the cells. Finally, he deemed it possible that bacteria underwent disintegration as the result of an acute infectious disease caused by an extraneous, ultramicroscopic virus.

Each one of these tentative explanations found a number of supporters among those subsequently studying the phenomenon. Our investigations, however, have suggested still another explanation of the appearances, namely, that they are due to the rupture of the bacterial cell wall which is unable to withstand the rise in internal pressure caused by the imbibition of water. The fact that the cytoplasm is quickly dissolved in the surrounding medium after the bursting of the bacteria indicates that the intracellular contents have been rendered soluble through some process of digestion prior to the bursting. Granting this to be true, the lysed cultures should show the presence of the products of digestion of the bacterial cytoplasm. Attempts have been made unsuccessfully in the past to find the products of hydrolysis of bacterial protein in lysed cultures. It seems possible that the failure was due to the fact that lysis was

carried out in beef infusion broth—a medium so rich in various protein-split products that a small increase caused by the hydrolysis of bacterial protein might have been masked. In our experiments here to be reported the lysis was made to take place in synthetic media, free from organic nitrogen.

EXPERIMENTAL.

Media.—Since the lysis of bacteria is conditioned by their free multiplication, it was first necessary to find synthetic media in which the bacteria which we intended to use, namely, *B. coli*, *B. pestis caviæ*, and a thermophilic bacillus respectively, would grow freely, and in which typical lysis would take place upon the addition of phage. After several trials we came upon two such media. The first, which represents a slight modification of the synthetic medium of Pozerski, and which was found to be suitable for *B. coli* and *B. pestis caviæ*, is prepared as follows:

Saccharose	20.0 gm.
Magnesium sulfate	1.0 "
Potassium sulfate	1.0 "
Disodium phosphate (anh.)	1.0 "
Ammonium succinate	6.0 "
Distilled water	1000.0 cc.

The pH is adjusted to 7.3 before sterilization and the medium is autoclaved at 15 pounds pressure for 15 minutes. After sterilization the pH varied from 7.0 to 7.1.

The thermophilic bacillus was grown in a medium similar to the one proposed by Frankel, Barber, and Pyle,[1] the composition of which is:

Ferric chloride	0.001 gm.
Magnesium sulfate	0.001 "
Calcium chloride	0.001 "
Dextrose	10.000 "
Disodium phosphate (anh.)	7.000 "
Dipotassium " "	9.000 "
Ammonium acetate	1.400 "
Distilled water	1000.0 cc.

The pH is adjusted to 7.0 with 10 per cent phosphoric acid. Sterilization is by autoclave at 15 pounds pressure for 15 minutes.

Chemical Methods.—After a suitable period of growth, all cultures were subjected to the same treatment. 20 gm. of hydrated barium hydroxide were added to each liter of medium. The volume of the liquid was reduced to 100 cc. by distillation under diminished pressure from a water bath kept at a temperature

[1] Frankel, Barber, and Pyle, *J. Infect. Dis.*, 1921, xxiv, 9.

between 60° and 70°C. At this point the material was tested for ammonia by Nessler's reagent. If ammonia was still present, sufficient 5.0 per cent barium hydroxide solution to give a slight excess of alkali together with 500 cc. of distilled water was added, and the distillation was continued until the Nessler's reagent gave a negative test. The precipitate of barium salts was then removed by filtration, and the precipitate thoroughly washed. The barium remaining in the filtrate and washings was removed by carefully adding dilute sulfuric acid. The reaction was made slightly alkaline to litmus by dilute sodium hydroxide, and again distilled to a volume of 100 cc. The precipitate of barium sulfate was removed by filtration or by centrifuging and then washed. The filtrate and washings were distilled as before and washed into a 50 cc. volumetric flask, to

TABLE I.

	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$		Increase of $\frac{NH_2}{N_2}$ ratio in the presence of phage
	Control	Phage	Control	Phage	Control	Phage	
Column	1	2	3	4	5	6	7
	gm.	*gm.*	*gm.*	*gm.*			*per cent*
B. coli	0.00248	0.00206	0.000842	0.000842	0.339	0.408	20.3
" "	0.00700	0.00707	0.00155	0.00183	0.221	0.259	17.2
" "	0.00686	0.00700	0.00140	0.00168	0.205	0.241	17.5
" *pestis caviæ*	0.00945	0.00875	0.00325	0.00553	0.344	0.632	83.7
" " "	0.00794	0.00770	0.00232	0.00383	0.293	0.497	69.7
" " "	0.00472	0.00490	0.00155	0.00291	0.329	0.595	80.8
Thermophile	0.00805	0.00770	0.001725	0.001725	0.214	0.224	4.58
"	0.00630	0.00630	0.001390	0.001668	0.221	0.265	20.2
"	0.005425	0.005775	0.000973	0.001390	0.180	0.241	39.6

which sufficient acetic acid had been added to make the solution acid. The total nitrogen in this filtrate was determined on 10 or 20 cc. aliquots by the Kjeldahl method. Amino nitrogen was determined by the method of Van Slyke

General Procedure.

All cultures used in the experiments, as well as the lytic filtrates employed, were carried through a number of passages on appropriate synthetic media previous to their use in the tests described below.

The first series of tests was made with the idea of determining any difference in the free amino acid content between cultures containing phage and those without phage.

TABLE

	18 hr. period. Before addition of phage					
	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_3}{\text{Total } N_3}$	
	Control culture	Culture to which phage is to be added	Control culture	Culture to which phage is to be added	Control culture	Culture which pha is to be added
Column	1	2	3	4	5	6
	gm.	*gm.*	*gm.*	*gm.*		
B. pestis caviæ	0.002975	0.002975	0.0006356	0.0006356	0.214	0.214
" " "	0.002800	0.002800	0.0005520	0.0005520	0.197	0.197
" *coli*	0.001750	0.001750	0.000349	0.0003490	0.199	0.199
" "	0.002275	0.002275	0.0004147	0.0004147	0.182	0.182

TABL

	18 hr. period. Before the second addition of bacteria						
	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_3}{\text{Total } N_3}$		Increase of the $\frac{NH_3}{N_3}$ ratio in the culture with phage $\left(\frac{\text{Column 6}-\text{Column 5}}{\text{Column 5}}\right)$
	Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage	
Column	1	2	3	4	5	6	7
	gm.	*gm.*	*gm.*	*gm.*			*per cent*
Experiment I	0.00350	0.00315	0.000281	0.000280	0.0804	0.0889	10.57
" II	0.00263	0.00245	0.000281	0.000351	0.107	0.1430	33.70

II.

24 hrs. after addition of phage. Total of 42 hrs.								
Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$		Increase of the $\frac{NH_2}{N_2}$ ratio in the culture with phage $\left(\frac{\text{Column 12}-\text{Column 11}}{\text{Column 11}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the control during second period of incubation $\left(\frac{\text{Column 11}-\text{Column 5}}{\text{Column 5}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the presence of phage during second period of incubation $\left(\frac{\text{Column 12}-\text{Column 6}}{\text{Column 6}}\right)$
Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage			
7	8	9	10	11	12	13	14	15
gm.	gm.	gm.	gm.			per cent	per cent	per cent
004725	0.00560	0.001400	0.00203	0.2963	0.3625	22.3	38.4	69.4
005950	0.00577	0.001242	0.001656	0.2087	0.2867	37.3	10.6	45.2
005770	0.00245	0.001576	0.000685	0.2729	0.2797	2.4	37.1	40.5
005075	0.00245	0.001380	0.000828	0.2719	0.3379	24.3	49.4	85.6

III.

6 hrs. after the second addition of bacteria. (Total period 24 hrs.)								
otal nitrogen per 000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$		Increase of the $\frac{NH_2}{N_2}$ ratio in the culture with phage $\left(\frac{\text{Column 13}-\text{Column 12}}{\text{Column 12}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the control during the second period of incubation $\left(\frac{\text{Column 12}-\text{Column 5}}{\text{Column 5}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the presence of phage during second period of incubation $\left(\frac{\text{Column 13}-\text{Column 6}}{\text{Column 6}}\right)$
ontrol ulture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage			
8	9	10	11	12	13	14	15	16
gm.	gm.	gm.	gm.			per cent	per cent	per cent
00455	0.00420	0.000557	0.000697	0.1224	0.1658	35.4	52.1	86.5
0560	0.00612	0.000826	0.001380	0.147	0.202	37.4	37.4	41.2

In each experiment two flasks of 1 liter each, containing precisely the same medium, were used, and each was inoculated with 1 cc. of an 18 hour culture of bacteria grown in the synthetic medium. To one of the flasks was added 0.5 cc. of phage, also grown on synthetic medium—the phage titer varying between 10^{-7} and 10^{-9}. The other flask served as a control.

The cultures of *B. coli* and *B. pestis caviæ* were incubated for 40 to 48 hours at 37°C., while the thermophilic bacillus was grown for 24 hours at 48°C. After the period of growth the cultures were treated as previously described.

Table I gives the results of these experiments. A comparison of Columns 5 and 6 shows that the amount of amino acids in the cultures containing phage is much larger than that in the control cultures.

The direct source of the increase in amino nitrogen in the presence of phage is not immediately apparent. The accumulation of amino nitrogen may be the result of increased hydrolysis of bacterial protein. It is possible, however, that, owing to a marked diminution in the numbers of live bacteria resulting from active lysis, the utilization of amino nitrogen present in the medium as the result of normal autolysis, is much slower in the flask containing phage than in the control. Such a condition would also lead to the accumulation of amino nitrogen in the flask with phage.

To determine the point thus brought up, the following experiment was performed:

Two flasks, each containing 2 liters of medium, were inoculated with 2 cc. of an 18 hour culture of bacteria and incubated for 18 hours. At this time 1 liter of the culture was removed from each flask for analysis (Table II, Columns 1 to 6), 1 liter of culture being retained in each flask. To one of the flasks 10 cc. of purified (amino nitrogen-free) bacteriophage were added; the other flask received 10 cc. of the same phage inactivated by heat and served as a control. After a further period of 24 hours of incubation at 37°C., the contents of both flasks were analyzed (Table II, Columns 7 to 12).

From a comparison of the data recorded in Table II (Columns 5 and 11), it appears that in the absence of bacteriophage, the concentration of amino nitrogen continues to increase on prolonged incubation of cultures. Thus (Column 14), if the effect of phage was merely to bring about destruction of bacteria, the result would have been to lower the rate of accumulation of amino nitrogen. The actual observation (see Table I, Columns 5 and 6, and Table II, Columns

11 and 12) shows, however, that in the presence of active lysis, amino nitrogen accumulates in the culture containing the phage considerably faster than in the control culture (compare Columns 14 and 5). These findings indicate that phage increases the rate of hydrolysis of bacterial protein.

It was thought that this point could be further elucidated by adding quantities of young, living bacteria to test cultures, and determining the effect upon the amino nitrogen content.

2 cc. of an 18 hour culture of *B. pestis caviæ* were added to each of two flasks containing 2 liters of the synthetic medium. In addition, 0.5 cc. phage (titer 10^{-8}) was added to one of them. After incubation for 18 hours, 1 liter was taken from each and analyzed as before (Table III, Columns 1 to 6). Equal amounts of a heavy suspension of young bacteria in synthetic medium, obtained by centrifuging the organisms from 2 liters of an 18 hour culture, were added to the remaining portions of the test cultures. 6 hours later these cultures were analyzed (Table III, Columns 8 to 13).

By comparing Columns 14 and 7, it is readily seen that after the addition of large numbers of living bacteria, the free amino acid content increases, and the increase is greater in those cultures containing phage. These results strengthen the conclusion drawn from preceding tests, namely, that lysis of bacteria by phage is accompanied by hydrolysis of bacterial protein.

CONCLUSIONS.

1. During the process of lysis by bacteriophage, there is an appreciable increase in the amount of free amino acid present in the culture.

2. The increase of free amino acid is due to hydrolysis of bacterial protein.

STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

X. Toxin Production by Normal and by Phage-Resistant Shiga Dysentery Bacilli.

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That the administration of bacteriophage during the course of an infectious disease frequently fails to affect the course of the infection is a common finding. One of the principal reasons assigned for this is the development of a race of bacteria resistant to lysis. Since it is well recognized that the resistant bacteria often show marked divergences from the type in many of their biological characteristics, it becomes of importance to know if their ability to produce disease is also different. There have been few studies of this question.

The commonly accepted view is that resistant bacteria are also characterized by an increased virulence. D'Hérelle (1) makes the general statement that bacteria resistant to the bacteriophage show an enhanced virulence, and then extends this, saying (2) "the degree of virulence of a bacterium is strictly in relation with its degree of resistance to the bacteriophage." Later (3), he states that susceptible *B. coli* is harmless, and that when pathogenic, it is the result of its resistance to phage, but he gives no experimental data in support of this contention.

In studying *B. pestis*, d'Hérelle (4) found that while the susceptible organism kills a guinea pig in a dose of 0.1 cc., the resistant strain kills in a dose of 0.0002 cc. Quiroga (5) obtained a resistant strain of *B. pyocyaneus*, of which a loopful injected intravenously killed in 24 hours, while a loopful of the susceptible strain killed in 3 days. Gratia (6) described a resistant strain of *B. coli* that was highly virulent for guinea pigs, not subject to phagocytosis, and that caused septicemia; while the susceptible strain was weakly virulent, subject to phagocytosis, and caused purulent peritonitis. Kauffmann (7) described resistant (*schleimige*) strains of *B. coli* characterized by greatly increased virulence for rabbits and guinea pigs and by resistance to phagocytosis. Wollstein (8) described a resistant strain of Shiga dysentery bacilli that would kill rabbits in 1/5 the dose required for the susceptible strain. Dutton (9), working with streptococci, says that the

outcome of disease depends upon the balance between the virulence of the bacteriophage and the virulence of the bacterium. He, however, considers all streptococci to be mixed with bacteriophage, so that virulence of the bacteria in this case indicates their ability to survive, and not variation in any other sense.

All of the reported studies were made with suspensions of the bacteria and concerned their ability to kill animals; none was related to toxin production. We therefore undertook the study of toxin production by *B. dysenteriæ* Shiga, an organism that causes disease by the elaboration of toxin and not by invasion.

Olitsky and Kligler (10) described the production of two types of toxin by the Shiga dysentery bacillus. The exotoxin acts on the nervous system, determining a paralysis, while the endotoxin acts on the intestinal tract, causing diarrhea and hemorrhages in the intestinal wall. Their methods were followed in our studies, and while a complete separation of the effects of the two toxins was not usually obtained, the predominance of those of one type over the other, depending on the method of preparation, was ordinarily found. In additional experiments an anti-exotoxin was also used for purposes of separation, but the results did not differ from those secured by other methods, so the protocols are not included. An exact quantitative study of toxin production was not attempted, as it sufficed for the object of the experiments to detect whether loss or great increase of this property had occurred. Only the time of death is recorded in the tables, but not only the symptoms but the autopsy findings were characteristic for the type of toxin used. The tables are each typical of experiments that were repeated several times.

EXPERIMENTAL.

B. dysenteriæ Shiga 109 and Laudman phage, active on it to a titer of 10^{-9}, were used.

Resistant bacteria were isolated by continuing incubation after lysis in broth until an overgrowth of resistant bacteria occurred. This was streaked in a Petri dish, and restreaked each day from single colonies for six generations, and then inoculated on an agar slant. The resistant strain thus secured was shown to be resistant to lysis and free from phage before being used.

Exotoxin was prepared by growing the bacteria in broth for 5 days at 37°C. and then filtering through a Berkefeld V candle. This sterile material was injected intravenously into rabbits (Table I).

The filtrates from the normal and from the resistant cultures resulted in similar symptoms and autopsy findings, and the severity of the effects was roughly proportional to the dose administered. The variation in the time of death was within the ordinary limits of individual variation.

TABLE I.

The Production of Exotoxin.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 3–94 1110 gm.	Normal	0.75	Died in 18 hrs.
No. 3–44 1300 gm.	Normal	0.3	Died in 64 hrs.
No. 3–49 1325 gm.	Normal	0.3	Died in 130 hrs.
No. 3–95 1100 gm.	Resistant	0.75	Died in 29 hrs.
No. 3–46 1400 gm.	Resistant	0.3	Died in 52 hrs.
No. 4–07 3125 gm.	Resistant	0.3	Died in 55 hrs.

TABLE II.

The Production of Endotoxin.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 3–43 930 gm.	Normal	0.75	Died in 18 hrs.
No. 3–48 1175 gm.	Normal	0.3	Dead in 40 hrs.
No. 3–93 710 gm.	Resistant	0.75	Dead in 18 hrs.
No. 3–50 1160 gm.	Resistant	0.3	Dead in 93 hrs.

Endotoxin was prepared by the method of Olitsky and Kligler (10). The 24 hour growth of the bacteria in Blake bottles was washed off in saline solution (15 cc. to each bottle) and incubated for 3 days at 37°C., and then filtered through a Berkefeld V candle. The filtrate

thus secured was heated to 80°C. for 1 hour before intravenous injection. As the results show (Table II), there was no appreciable difference in the production of endotoxin by the normal and by the resistant strains.

After the completion of the first series of experiments, it was found that the resistant organism showed a tendency to revert to susceptibility. This was not evidenced by visible lysis, but by the fact that when a diluted phage was incubated with the bacterium, it would increase in titer. Recourse was then had to growing the resistant bacteria in the presence of phage to prevent reversion. Toxin preparations obtained from organisms so treated had the same effects as those preparations of which the results are recorded in the previous tables.

TABLE III.

Examination of the Resistant Strain.

Bacteria inoculated	0	Normal Shiga	Resistant Shiga	Resistant Shiga after 10 daily transfers
Laudman phage diluted 1–10,000	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.
Titer before incubation	10^{-3}	10^{-3}	10^{-3}	10^{-3}
Incubated 48 hrs. Heated to 56°C. for 50 min. Titrated by Appelmans' method.				
Titer	10^{-3}	10^{-9}	10^{-3}	10^{-3}

The series of experiments was again repeated, with freshly isolated resistants. After isolating them in the usual manner and demonstrating that they were not subject to visible lysis and did not carry phage, they were incubated with diluted phage and shown to be unsuitable for its increase. As a further control, the strain was carried through repeated transfers in broth and tests showed that it had not reverted sufficiently to serve for the increase of phage. The results of these tests are given in Table III. This eliminates any doubt of the resistance of the bacteria used, but as a further control, the tests were repeated on bacteria taken from each flask just prior to filtration.

Exotoxin was prepared in the manner previously described. *Endotoxin* was prepared according to the method of McCartney and Olitsky (11), the method

being varied by using bacteria grown overnight on the agar surface in a Blake bottle. The growth was suspended in 25 cc. of saline for each bottle. 10 cc. of this were centrifuged and washed once in saline and then suspended in 5 cc. of 1 per cent sodium carbonate. This was sealed in an ampoule, heated to 56°C.

TABLE IV.

Exotoxin Production by Bacteria Completely Resistant.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
A 195 1950 gm.	Normal	0.5	Dead in 72 hrs.
A 194 1985 gm.	Normal	1	Dead in 24 hrs.
A 190 1975 gm.	Normal	2	Dead in 48 hrs.
A 193 2050 gm.	Resistant	0.5	Dead in 48 hrs.
A 196 2060 gm.	Resistant	1	Dead in 24 hrs.
A 192 2025 gm.	Resistant	2	Dead in 24 hrs.

TABLE V.

Production of Endotoxin by Bacteria Completely Resistant.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 25 620 gm.	Normal	0.05	Dead in 72 hrs.
No. 29 500 gm.	Normal	0.025	Dead in 96 hrs.
No. 26 610 gm.	Resistant	0.05	Dead in 48 hrs.
No. 27 560 gm.	Resistant	0.025	Dead in 72 hrs.

for 1 hour, and incubated overnight. The resulting fluid was very viscous; it was injected intravenously after cultures had shown it to be sterile (Table V).

The results of these experiments (Tables IV and V), as of the previous ones, show that from the standpoint of toxin production, the normal and the resistant bacteria are identical.

DISCUSSION.

The failure of Shiga dysentery bacilli to show an alteration in toxin production with the development of resistance to the bacteriophage is at variance with the commonly accepted view. While an increased virulence has not always been found in such instances, an alteration has usually been reported. Fejgin (12) described three strains of phage-resistant Shiga dysentery bacilli showing a loss of toxin production to such an extent that 4 to 5 cc. of a 24 hour broth culture failed to produce characteristic symptoms in a rabbit when injected subcutaneously. Blair (13), by the action of bacteriophage, secured two strains of diphtheria bacilli that were atoxic for guinea pigs. Only one of these, however, was resistant to phage. In this laboratory it was found (14, 15) that resistant strains of *B. pestis caviæ* were avirulent, and that virulence returned with the reversion to susceptibility.

The experimental data presented and the review of the literature show that the relation existing between resistance to bacteriophage and toxin production or virulence is by no means a constant one. Furthermore, it is a well recognized fact that bacteria resistant to phage may show quite pronounced differences from the ordinary in other characteristics. The most logical explanation of these facts seems to be that the properties of resistance to phage, and of toxin production are two independent manifestations of bacterial variation or dissociation. If this is correct, it should be possible, by examining a sufficiently large number of strains, to secure races showing all possible combinations in the degree of these two properties.

SUMMARY.

1. The production of exotoxin and of endotoxin by normal Shiga dysentery bacilli and by strains resistant to Laudman phage was found to be the same.

2. The presence of phage did not alter toxin production by the resistant organism.

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THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

IV. The Malignant Fibroblast of Jensen Sarcoma.

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Plate 8.

(Received for publication, May 18, 1928.)

In a preceding article,[1] it was shown that the malignant fibroblasts of Sarcoma No. 10 of the Crocker Foundation differ from the normal cell type in certain physiological properties which are undoubtedly responsible for their malignancy. In order to ascertain whether these properties are of general significance, an analysis must be made of the behavior of the active element of as many experimental tumors as possible. Some hidden characteristics common to all tumor cells may then become apparent. The purpose of the experiments described in the present paper was to study the fibroblasts of Jensen sarcoma when they are isolated in pure culture, and to compare them with normal fibroblasts and with the malignant cells of Sarcoma No. 10.

Isolation of a Strain of Malignant Fibroblasts from Jensen Sarcoma.

Jensen sarcoma has been studied by many investigators, and its characteristics are well known. A fragment of this tumor, when sectioned and stained, appears to be composed mainly of fusiform cells with large clear nuclei. Between the cells are seen the darker nuclei of macrophages. These macrophages are about one-sixth less numerous than the fibroblasts. The tumor is easily propagated by transplantation to rats, and kills the animals without producing any metastases.

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, xlviii, 105.

An active strain of Jensen sarcoma was obtained by us from the Crocker Foundation, through the kindness of Dr. Woglom. On January 6, 1928, a few fragments of a tumor freshly extirpated from a rat were cultivated in three D-3 flasks containing a solid medium composed exclusively of diluted chicken plasma. After coagulation, the clot was washed twice with Tyrode solution and the fluid medium introduced. The medium in the first flask consisted of rat serum, in the second of chick embryo juice, and in the third of Tyrode solution. During the first 24 hours of incubation, the tissue fragments surrounded themselves with macrophages, but these cells were present in a much larger number around the tissues cultivated in serum than around those cultivated in embryo juice. After a few days, the cultures made in embryo juice were composed almost exclusively of fibroblasts, and the area covered by the migrating cells was not very extensive. From the tissues cultivated in rat serum and in Tyrode solution, a large number of macrophages and fibroblasts migrated. After about 10 days, the cultures were transferred to other flasks and an attempt was made to isolate a strain of fibroblasts and a strain of macrophages by cultivating the tissues in different media. One of the media was composed exclusively of rat serum, and the other of chick embryo juice. Most of the tissues fed exclusively on embryo proteins died. The fragments cultivated in rat serum, on the contrary, grew actively, and after about a month a pure strain of cells resembling fibroblasts was obtained. The wandering cells completely disappeared. Each colony of fibroblasts was allowed to grow for about 8 or 10 days in the solid medium. Then the coagulum was removed from the flask and the tissues were extirpated and embedded in a fresh coagulum in another flask.

Inoculations into rats of some of the pure cultures of fibroblasts during February, March, and April, 1928, were followed in about 10 days by the appearance of a small tumor which grew rapidly later. The fibroblast-like cells were obviously malignant.

Morphological Characteristics of the Malignant Cells.

The cytological study of the cultures has been made according to the technique described in a previous article.[2] The colonies are composed

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 261.

of spindle-shaped cells, with a large nucleus and a refringent cytoplasm. Instead of sending forth fan-like processes, as normal and sarcomatous fibroblasts often do, these cells are strictly fusiform, and rarely present any lateral branches (Fig. 1). Their size is equal to that of normal fibroblasts. The surface of the cells averages 1,456 sq. μ, and that of the nucleus 205 sq. μ. The nucleus is broad and short, and contains one or two nucleoli. The segregation apparatus is composed of small, dust-like vesicles. No degeneration vacuoles were observed. The mitochondria are slightly shorter and larger than those of normal cells. There is no abnormal mitosis. Very few binucleate cells are seen. None of the nuclear or cytoplasmic structures show any evidence of degeneration. There are no dead cells in the cultures. It is evident that this malignant element of Jensen sarcoma is a healthy cell.

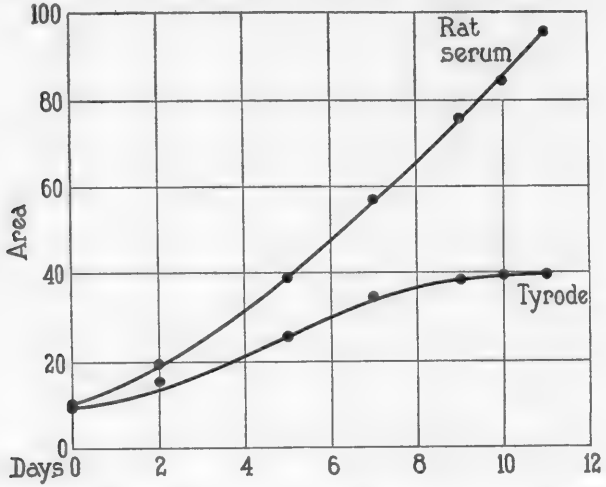
Architecture of the Colonies.

The malignant cells congregate in a tissue which is not very dense. They have some tendency to scatter. Their processes do not form an intricate network as in the colonies of normal and Sarcoma No. 10 fibroblasts. The appearance of the tissue is loose. The Jensen colonies are typically fibroblastic in structure, although they may slightly resemble certain communities of macrophages. They are round or oval in shape, less extensive, and more transparent than those of Sarcoma No. 10. From the outer edge of the tissue, many cells wander freely into the surrounding medium. However, they remain bipolar and fibroblast-like, and never assume the appearance of macrophages. From the point of view of their texture, the colonies vary in some measure according to the nature of the medium. In rat serum, they are denser than in chick embryo juice or in calf liver digest. After several months of life in chick embryo juice, some cultures have become thicker and assumed an appearance practically similar to that of Sarcoma No. 10.

Residual Growth Energy.

The residual growth energy of Jensen fibroblasts was tested by the duration of the life and the extent of the growth of the colonies in a

medium composed of Tyrode solution (Text-fig. 1). Four experiments were made, as summarized in Table I. The duration of the life



TEXT-FIG. 1. Experiment 6855-C. Residual activity of Jensen sarcoma fibroblasts.

TABLE I.

Residual Energy of Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of life	Relative increase
		days	
1	6855-C	10	3.20
2	10356-D	19	2.16
3	10356-D	19	2.43
4	10356-D	19	4.32
Average.....		16.8	3.03

of colonies in Tyrode solution did not exceed 17 days. The relative increase was 3. It appears that Jensen sarcoma cells have a little more capacity for storing food than normal cells.

Rate and Duration of the Growth.

The rate of growth of the strain was ascertained in the usual way by measuring the area of the colonies cultivated in a nutrient medium.[3] In Table II, the results of fifteen experiments are summarized, where the relative increases in nutrient media during a passage of several days were compared for 6 to 17 days in the course of a few passages. The area of the colonies may increase about five times in 6 days. The rate of growth varies a great deal, depending on the previous condition of the strain and some other factors. The duration of the life of the tissue in a nutrient medium would appear to be unlimited. After 4 months of life *in vitro*, the malignant fibroblasts proliferate as actively as at the beginning of their cultivation. The inoculation of such cultures into rats produces a tumor in a few days. Therefore, it seems probable that they can live indefinitely *in vitro* without losing their malignancy.

Action of Jensen Fibroblasts on Their Medium.

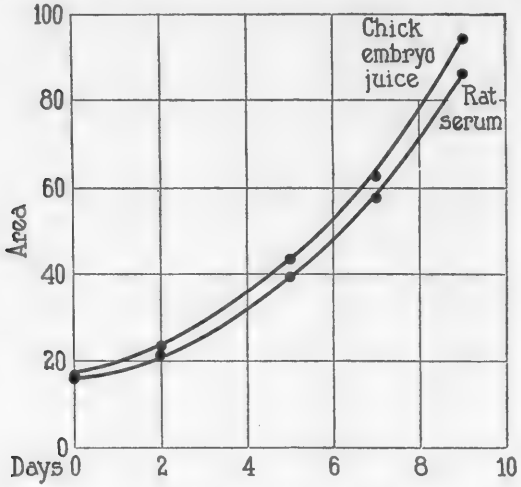
1. Liquefaction of the Fibrin.—When sarcomatous cells were cultivated in washed chicken plasma, no digestion of the coagulum ever occurred. The medium remained homogeneous. When cultivated in rat plasma, the cells digested the coagulum, as happened in the cultures of Sarcoma No. 10 and of Rous sarcoma.

2. Acid Production.—Fragments of fresh tumor and of fibroblasts in pure culture were embedded in diluted chicken plasma, coagulated with embryo juice, and stained with phenol red, as with Sarcoma No. 10. After a few hours, the sarcomatous colonies appeared as golden yellow spots on the pink background of the medium.

Food Requirements.

Jensen fibroblasts multiplied readily in rat serum (Table II, Text-fig. 2). Colonies have been kept in a mixture of rat serum and Tyrode solution for 4 months, and are still growing with unabated activity. They did not develop in chicken serum (Table III, Text-fig. 3). When the malignant fibroblasts were placed in chick embryo juice, they grew

[3] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.



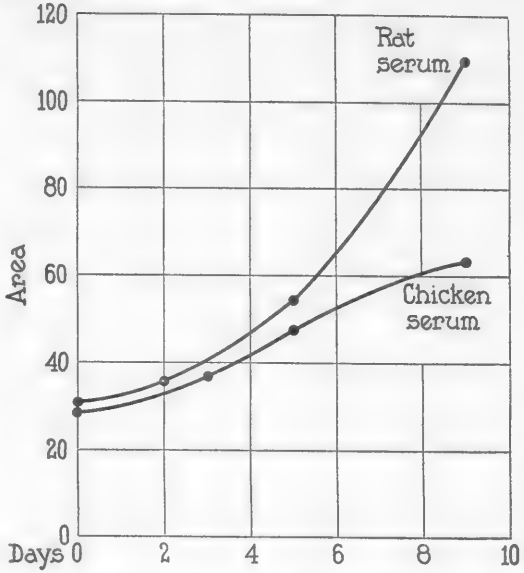
TEXT-FIG. 2. Experiment 10322-D. Effect of rat serum and chick embryo juice on Jensen sarcoma fibroblasts.

TABLE II.

Effect of Rat Serum and Chick Embryo Juice on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	No. of passages	Relative increase during last passage		Ratio: $\frac{E}{C}$
				Control in rat serum	Experiment in chick embryo juice	
		days				
1	10322-D	9	1	4.18	4.65	1.11
2	10322-D	9	1	4.52	4.70	1.04
3	10370-D	17	2	2.20	2.50	1.01
4	10370-D	17	2	3.00	3.20	1.07
5	6688-C	7	1	5.10	5.40	1.06
6	234-J	8	1	3.70	4.90	1.32
7	10364-D	17	2	1.80	1.85	1.04
8	10439-D	7	1	2.54	2.45	0.96
9	10439-D	7	1	2.85	2.76	0.97
10	1691-H	6	1	5.14	5.30	1.03
11	10327-D	17	2	4.44	4.00	0.90
12	10327-D	17	2	4.00	3.46	0.87
13	10380-D	9	1	4.35	3.80	0.87
14	10421-D	23	3	1.20	1.44	1.20
15	10421-D	23	3	1.00	1.00	1.00

more slowly. In some cultures, the growth stopped altogether and the colonies died. However, the tissue became accustomed to the



TEXT-FIG. 3. Experiment 10426-D. Effect of rat serum and chicken serum on Jensen sarcoma fibroblasts.

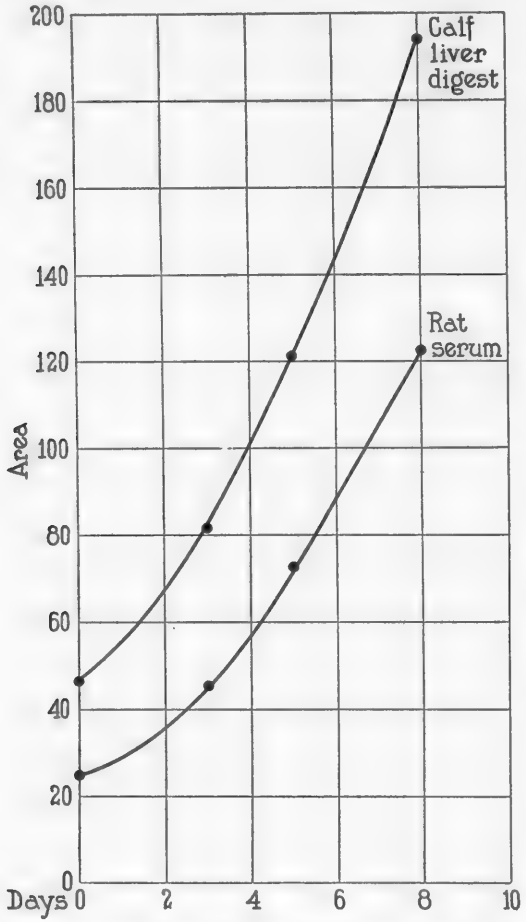
TABLE III.

Effect of Rat Serum and Chicken Serum on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	Control in rat serum	Experiment in chicken serum	Ratio: $\frac{E}{C}$
		days			
1	10375-D	10	3.80	1.10	0.29
2	10375-D	10	4.00	3.70	0.93
3	10426-D	9	3.57	1.20	0.34
4	10427-D	9	2.57	1.03	0.40
Average.....		9.5	3.49	1.76	0.49

medium, and finally grew as actively in embryo tissue juice as in serum. A comparison was made of the rate of growth of the malignant fibro-

blasts in diluted embryo juice and in rat serum (Table II, Text-fig. 2). Calf liver digests, prepared according to a technique previously de-



TEXT-FIG. 4. Experiment 348-J. Effect of rat serum and calf liver digest on Jensen sarcoma fibroblasts.

scribed,[4] were used at concentrations of 1:8 and 1:32 as a food for Jensen colonies. No marked differences were observed in the effect

[4] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371.

TABLE IV.

Effect of Rat Serum and Calf Liver Digest (1:32) on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	No. of passages	Relative increase during last passage		Ratio: $\frac{E}{C}$
				Control in rat serum	Experiment in calf liver digest	
		days				
1	10345-D	10	1	4.40	5.00	1.14
2	10345-D	10	1	3.60	5.80	1.61
3	10345-D	10	1	3.40	5.50	1.62
4	289-J	8	1	3.08	9.99	3.24
5	288-J	8	1	4.44	8.60	1.94
6	10398-D	19	2	2.64	4.80	1.82
7	10398-D	19	2	3.00	3.75	1.25
8	10398-D	19	2	1.52	3.64	2.40
9	10398-D	19	2	2.00	4.29	2.15
10	10460-D	29	3	2.73	4.00	1.47
11	10460-D	29	3	2.65	3.09	1.15
12	10460-D	29	3	2.95	3.57	1.21
13	10460-D	29	3	3.05	4.00	1.31
14	10516-D	35	4	1.90	4.00	2.10
15	10516-D	35	4	3.23	5.67	1.76

TABLE V.

Effect of Rat Bone Marrow on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	Relative increase		Ratio: $\frac{E}{C}$
			Control without bone marrow	Experiment with bone marrow	
		days			
1	1877-H	8	3.30	3.67	1.11
2	1877-H	8	2.20	3.30	1.50
3	1877-H	8	3.00	4.60	1.53
4*	10366-D	13	2.60	4.33	1.67
Average.....		9.25	2.76	3.68	1.45

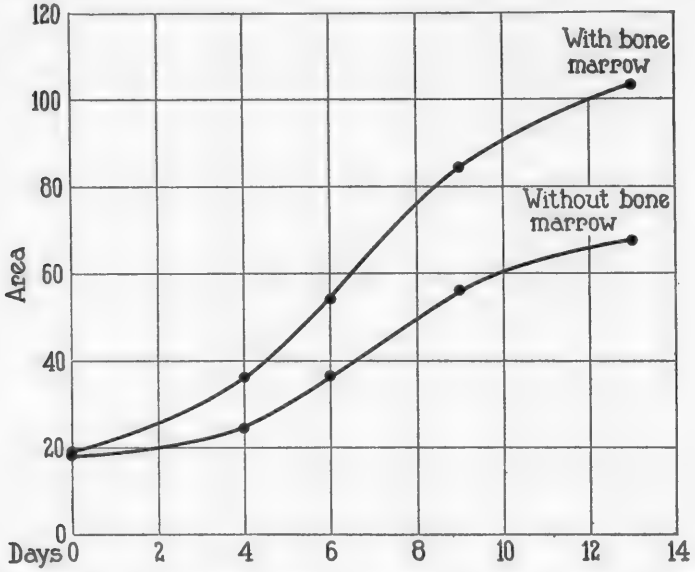
* Fluid medium composed of rat serum.

of these solutions. The results of one series of experiments are summarized in Table IV. Calf liver digest appears to be a better nutrient medium for sarcoma cells than serum (Text-fig 4). It has supported

their proliferation for a long time. After 35 days cultivation in such a medium, the sarcoma fibroblasts proliferate with undiminished rapidity.

Effect of Bone Marrow on Jensen Fibroblasts.

A culture of Jensen fibroblasts was divided into two equal parts. The halves were embedded at the opposite sides of a D-3 flask in a



TEXT-FIG. 5. Experiment 10366-D. Effect of rat bone marrow on Jensen sarcoma fibroblasts.

coagulum of chicken plasma. Close to one of the fragments was placed a bit of rat bone marrow. The fluid medium consisted of Tyrode solution in three experiments, and of rat serum in one. The measurement of the growth of both colonies of fibroblasts was made according to the ordinary technique.[3] The results of the experiments, summarized in Table V, show that the presence of the wandering cells from the bone marrow definitely activated the multiplication of the Jensen fibroblasts (Text-fig. 5).

DISCUSSION.

A pure strain of fibroblast-like cells, obtained from a fragment of Jensen sarcoma, has maintained its malignancy unimpaired after several months of life *in vitro*. The element which is the carrier of the malignant characteristics appears to be a true fibroblast, from a morphological point of view. It differs from the rat fibroblasts observed in pure cultures by the refringement aspects of the cytoplasm and its coarseness. The cell is generally bipolar and does not send out any fan-like processes (Fig. 1). Instead of being long and slender as in the normal cells, the nucleus is more globular. While the projected areas of the cell and nucleus of the normal type are respectively 1,960 and 189 sq. μ, those of the Jensen cell are 1,456 and 205 sq. μ. The size of the nucleus is relatively larger in the neoplastic than in the normal cell. The small segregation apparatus is composed of dust-like vesicles and a large number of very small fat granules. No cytoplasmic inclusions or degeneration vacuoles are observed. The mitochondria are shorter and larger than those of normal fibroblasts.

This cell is smaller than the malignant element of Sarcoma No. 10, which has a total area of 2,300 sq. μ, and a nuclear area of 230 sq. μ. Neither malignant cell shows any abnormality. They are healthy elements. The morphological characteristics, by which the specific properties of the cells may express themselves, have not as yet been discovered. It is probable that such characteristics will be detected when more elaborate techniques are used in the morphological investigation of the nucleus and the cytoplasm.

There is a striking difference between the Jensen sarcoma fibroblasts in pure cultures, and the elements composing this same tumor under the circumstances in which it has been studied by other workers. In order that the discrepancies in the results of the investigations may be correctly interpreted, it must be remembered that previous studies of Jensen sarcoma have been made on tissues just removed from the animals, or on fragments of tissues "cultivated" *in vitro* with the technique developed 15 years ago. According to this early procedure, which is still favored by many experimenters in this country and in Europe, a fragment of tissue is placed on a cover glass in a drop of plasma, and, under conditions that are uncontrolled and unknown, it

survives for a few days and degenerates. In this manner, Fell and Andrews,[5] after a careful cytological study of Jensen sarcoma, have recently described a number of abnormalities and degenerative phenomena, such as binucleate and multinucleate cells and abnormal mitoses. Similar observations had previously been made on sarcoma tissues by Lambert and Hanes.[6] Fell and Andrews[5] noted enlarged centrospheres, similar to those described by Lewis[7] in degenerating mesenchyme cells. They also observed epithelioid cells which differed from the fibroblast-like cells and from the wandering elements, and cells too that were greatly enlarged. These giant cells obviously came from the fibroblast-like cells. They usually contained one or two nuclei. The authors did not believe that there is any histiogenic relationship between those cells and the macrophage type which they also described. They supposed that the malignant component may be the macrophage, while the fibroblast represents the stroma element.

These abnormalities are evidently phenomena of secondary importance. They are not directly connected with the malignant characteristics of the cell, since typically malignant sarcoma fibroblasts, after they have lived for some months in pure culture, do not show any of these peculiarities. They must be attributed to the conditions which are brought about by the crowding of the cells, the setting free of toxic substances by the tissue itself, etc. It is evident that the mere observation of a fragment of tumor surviving *in vitro* for a few days, according to the procedure adopted by Fell and Andrews,[5] and other experimenters, is inadequate for ascertaining the characteristics which are specific of any cell type. For the fact is that the Jensen fibroblasts, properly cultured in the pure state, retain malignancy and do not show any striking morphological abnormalities which can be detected by the techniques used thus far. However, they differ from the normal fibroblasts by their relatively coarse structure and the special character of their colonies. The texture of the tissue which they form is not dense, although after a few months of growth *in vitro*

[5] Fell, H. B., and Andrews, J. A., *Brit. J. Exp. Path.*, 1927, viii, 413.

[6] Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiii, 495; xiv, 129; *J. Am. Med. Assn.*, 1911, lvi, 33, 791. Hanes, F. M., and Lambert, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1910–11, viii, 113.

[7] Lewis, W. H., *J. Exp. Med.*, 1920, xxxi, 275.

it becomes more compact. The cells have some tendency to scatter as macrophages do. But in spite of this characteristic, their colonies are typically fibroblastic, and do not resemble at all the groups of wandering cells scattered through a plasma coagulum. They may be distinguished at first sight from the colonies of Sarcoma No. 10 fibroblasts because they are less opaque, and their elements are less closely interwoven.

The residual growth energy of the Jensen fibroblasts is a little greater than that of normal fibroblasts and of the cells of Sarcoma No. 10. Their rate of growth in a nutrient medium does not differ markedly from that of these other cells. Like them they multiply *in vitro* to an unlimited degree. They feed upon chick embryo juice and on calf liver digest, as do normal fibroblasts and Sarcoma No. 10 fibroblasts. But they differ notably from both cells in that like macrophages they are able to grow indefinitely in rat serum. It is well known that normal fibroblasts die in a short time in such a medium; and Sarcoma No. 10 cells do not survive more than 10 days in diluted blood serum. The capacity of multiplying when placed in diluted serum is of great importance, as it enables the Jensen fibroblasts to grow within the interstitial lymph of the adult organism without the help of any other cell.

The Jensen fibroblasts differ from the normal type in two other respects. They liquefy coagulated rat plasma and produce a large amount of acid. It appears then that the power of dissolving the fibrin of a coagulum made of homologous plasma, and of producing an abnormal amount of acid, belongs to the malignant element of every sarcoma so far investigated: Rous sarcoma,[8] Sarcoma No. 10,[1] and Jensen sarcoma.

It is reasonable to suppose that the growth of Jensen sarcoma within the body is due to the property possessed by the malignant cells of feeding upon blood serum. Whereas normal fibroblasts do not multiply when in blood serum, the Jensen fibroblasts are enabled to proliferate in such a medium. Their malignancy within the organism may arise from this property. Such a difference in the food requirements of Jensen and normal fibroblasts is probably attributable to the increased acid production and proteolytic power of the tumor cell.

[8] Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.

CONCLUSIONS.

1. A pure strain of fibroblasts has been isolated from the Jensen rat sarcoma. The cells give rise to tumors on transplantation into animals and during several months of life *in vitro* have maintained their malignancy unchanged.

2. The malignant cells are generally coarser and more refringent than normal cells. They possess the cytological characteristics of fibroblasts without showing any morphological abnormality. They can be considered as healthy cells. The texture of their colonies is looser than that of normal fibroblasts and Sarcoma No. 10 fibroblasts. Their residual activity does not differ markedly from the normal. They proliferate unlimitedly in a nutrient medium.

3. They liquefy the fibrin of rat plasma and turn phenol red golden yellow. They do not liquefy the fibrin of chicken plasma.

4. They multiply in chick embryo juice, calf liver digest, and also in rat serum. Their growth activity is increased by the presence of bone marrow.

5. The unlimited growth of Jensen sarcoma within the body may possibly be attributed to the ability of the fibroblasts to maintain themselves upon the substances present in rat serum. This property itself probably depends upon the increased enzyme activity of the cells.

EXPLANATION OF PLATE 8.

FIG. 1. Camera lucida drawing of cells of Jensen fibroblasts. × 1,600.

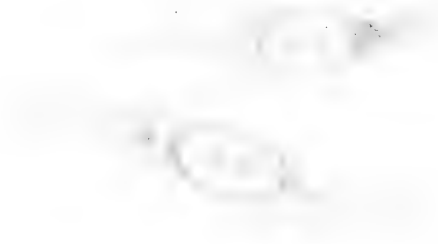


FIG. 1.

(Carrel and Ebeling: Fibroblast and macrophage. IV.)

RECIPROCAL AGGLUTINATION AND ABSORPTION OF AGGLUTININ TESTS WITH FIFTY-FOUR STRAINS OF HEMOLYTIC STREPTOCOCCI ASSOCIATED WITH AN EPIDEMIC OF PUERPERAL FEVER.

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INTRODUCTION.

In the late winter of 1927 an outbreak of puerperal fever occurred in the Sloane Hospital in New York City, reaching such proportions that the hospital had to be temporarily closed. In the course of 1 month 24 of 163 parturient women developed puerperal fever and 8 died, a mortality of 33 per cent. The epidemiological and clinical features of that epidemic will be reported elsewhere (1, 2). The purpose of this paper is to present the results of the biological and immunological study of 54 strains of hemolytic streptococci which were found to be associated with the epidemic.

This epidemic, like most others, was well under way before it was recognized as such. Two patients died with symptoms of sepsis from whom no cultures were taken and on whom no autopsies were performed. The third fatal case, at autopsy, showed streptococci in the peritoneal exudate. From the fourth, hemolytic streptococci were obtained by culture from the peritoneum after death. Thereafter it was recognized that an epidemic of hemolytic streptococcus puerperal fever was in full swing and cultures were made from the vaginæ and blood of all patients showing a post partum temperature above 101°F. Where death occurred cultures were also made at autopsy. Outside help was called upon to investigate the epidemic and the bacteriological research laboratory of the Department of Surgery undertook the study. There was some delay in organizing the work and it was not

until 18 days after the delivery of the first case of the series that a systematic bacteriological survey of the hospital was begun in an effort to discover if possible, the source of the infection. In the meanwhile a large group of nurses, one doctor and several patients had left the hospital, so that it was recognized that the survey could not be complete. In this survey it was found that the only places where the hemolytic streptococci could be found with any degree of constancy were the vaginæ of the patients and the noses and throats of some of the attending staff. The laundry, air and various objects in the delivery rooms and wards, the solutions and supplies yielded no hemolytic streptococci in culture. The noses and throats of the patients were entirely negative during and for some time after the epidemic. Altogether 54 strains were cultured, chiefly from the vagina, peritoneum and blood of patients and from the noses and throats of staff members. Two of these strains came from the vaginæ of patients not showing clinical symptoms of puerperal fever. These were picked up during the course of our routine examinations. All of the strains were studied with the view to determine if possible the biologic identity of two or more of these strains.

Krumwiede, Cooper and Provost, in their monograph on absorption of agglutinin (3), have clearly shown that this test is required to establish close biological similarity of bacterial antigens. Heterologous organisms will frequently agglutinate in a serum out of which they cannot completely absorb the agglutinin. The phenomenon of agglutination in such cases is supposed to be due to a group agglutinin. Heterologous strains which absorb all of the agglutinin out of a serum are much closer antigenically to the homologous strain than those which only absorb out the group agglutinin. In order to demonstrate that two organisms are exactly the same antigenically it is necessary to produce in animals an antiserum for each strain and then show that both organisms will not only agglutinate to the same degree in both sera but that they will both, in the same absorptive dose, completely absorb the agglutinin out of both sera. In other words there must be complete reciprocal agglutination and complete reciprocal absorption of agglutinin. Such a relationship brings very strong evidence in favor of the common origin of such strains, and probable sources of infection may be determined by this method. By this test, 2 years

ago, one of us, with Stevens was able to show that the last case in a series of hemolytic streptococcus wound infections was caused by an organism which was antigenically identical with a strain cultured from the unmasked nose of the instrument nurse assisting at that operation. It was further shown that the infecting strain was antigenically dissimilar to the strains cultured from the patient's own nose and throat and other strains cultured from the personnel of the operating room (4).

It was thought that such a study of the 54 strains from this epidemic of puerperal fever might indicate whether or not it was a true epidemic coming from a single source and whether or not the organism was being carried by any of the attending staff.

The sources of the 54 strains tested, were as follows: Four were cultured from patients' blood, twenty from patients' vagina, four from metastatic foci, one from the surface of a patient's thigh, one from the axillary abscess of a nurse who pricked her finger with a pin while taking care of one of the fatal cases, one from the peritoneum of a nurse who developed a primary hemolytic streptococcus peritonitis during the course of the epidemic, fourteen from the throats of staff members, seven from the noses of staff members, one from a patient's throat and one from a patient's nose. The two cultures from the patients' nose and throat were obtained in the course of the routine cultures carried out for several months after the epidemic was over and were included because they were the only positive cultures from the noses or throats of the parturient women. Two of the vaginal cultures and one from the patient's thigh were from two patients who did not show the clinical signs of puerperal fever. Three other nose cultures and eight other throat cultures from the staff on repeated transplantation in artificial media lost their hemolytic quality and were discarded.

EXPERIMENTAL.

Five strains from five different puerperal fever cases and one strain from the throat of one of the doctors who had been associated with several of the cases, were first taken for animal inoculation. Three of the patients' strains had been cultured from the blood and two from the vagina. The stock cultures were kept in cooked meat medium in which streptococci may survive for a year or more but they were transferred at intervals of 2 to 3 months. Out of the stock cultures they were transplanted into fresh meat medium for 24 hours and then were grown in phosphate buffered broth for 24 hours further. Rabbits were inoculated 3 days in succession every week with gradually increasing doses of whole culture, first heat killed and then living. A number of animals died of septicemia after the first small dose of living organisms. When the serum showed an agglutinating

titre of 1–2560, the animal was killed and the serum obtained. This generally required 2 months.

Agglutination tests were carried out in the usual way. The serum dilutions were made with phosphate broth and after the organisms were added, the dilutions ranged from 1–80 up to 1–2560. Most of the strains were granular at first but after being transplanted daily for a week or two in phosphate broth they became diffuse. Sterile potato was added to the phosphate broth for the final growth before the agglutination test. The tubes were incubated for 1 hour at 56°C. and the readings made at the end of that time. The reaction was almost always clear-cut and nothing seemed to be gained by keeping the test in the ice box overnight before reading, as advocated by some writers. In our records the degrees of agglutination ranged from ± to 4, while 0 denoted complete absence of agglutination.

TABLE I.

Preliminary Classification of 54 Strains in Main Agglutination Group.

Source	Total	Positive	Negative
Patients' blood	4	2	2
" vagina	20	14	6
" metastatic foci	4	4	0
" thigh	1	1	0
" nose	1	0	1
" throat	1	0	1
Staff, nose	7	3	4
" throat	14	4	10
" abscess	1	1	0
" peritoneum	1	1	0

The technique of absorption of agglutinin was similar to that used in our previous work (4) except that greater accuracy of dosage was attempted. The minimal absorptive dose had to be determined for each serum. This varied from 100 to 150 billion organisms for 1 mil of a 1–20 dilution of serum. After incubating in large flasks, the cultures were centrifuged and washed once in distilled water. They were then suspended in a concentrated form in distilled water and counted by means of a Gates turbidimeter (5). Compared with a known standard, the number of organisms per mil could be determined fairly accurately. Then twice the minimal absorptive dose of organisms was centrifuged and after pouring off the supernatant fluid, 1 mil of a 1–20 dilution of the serum to be tested was added and mixed with the organism sediment. The mixtures were then heated at 56°C. for 1 hour and shaken at quarter-hour intervals. At the end of that time the mixtures were placed in the ice box for the night. Next morning the serum was drawn off and tested for agglutination with the homologous strain, and the absorbing strain as described above.

Results.

A. The Results of the Preliminary Agglutination Tests.—(See Table I.) These preliminary tests seemed to indicate clearly that the great majority of the strains from the patients fell into one agglutinative group. The serum produced by the doctor's throat strain did not agglutinate any of the patients' strains and the organism did not agglutinate in any of the other sera. This seemed to show that the doctor's strain was definitely outside of the main group. Six of the vaginal strains and two of the blood culture strains (J and T) also seemed to be outside of the group for they showed no agglutination in any of the sera. One of these blood culture strains (J) had been used to produce one of the preliminary sera and this serum ran parallel with the other sera but failed to agglutinate the homologous strain. These discrepencies will be discussed below. When two or three strains came from the same patient they always reacted in a similar manner. The strain from the axillary abscess of one nurse and from the peritoneum of the other nurse fell into the main group, three strains from nurses' nose cultures and four from nurses' throat cultures were also included in the main group. These last seven strains came from six nurses, one yielding a positive culture in both nose and throat. At this stage, absorption of agglutinin tests might have been done but vacation intervened and during that time it was decided to produce fresh sera with a fair number of the strains which had been included in the preliminary agglutinative group.

Fourteen cultures were then selected for producing this new series of sera—three from nasal cultures and three from throat cultures of the six nurses and eight strains from the puerperal fever cases. The two blood culture Strains J and T which failed to agglutinate in the preliminary test were used along with six which had fallen into the preliminary agglutinating group. Sera were obtained for these fourteen strains by inoculating rabbits in the same way as before. As these sera were obtained they were tested against all of the patients' strains and all of the other strains which had been included in the preliminary agglutinating group.

B. Results of the Absorption of Agglutinin Tests with the Final Group of Fourteen Sera.—When three of the sera produced by the nurses' strains were absorbed by the patients' strains, it was found that the

TABLE II.

Agglutination and Absorption of Agglutinin Serum H, Produced by Nurse's Nose Strain H.

Tested with Strain H and 7 Patients' Strains.

Serum	Strain	Serum dilutions					
		1/80	1/160	1/320	1/640	1/1280	1/2560
Normal	H	0	0	0	0	0	0
"	R	0	0	0	0	0	0
"	L	0	0	0	0	0	0
"	E	0	0	0	0	0	0
"	Ma	0	0	0	0	0	0
"	Mc	0	0	0	0	0	0
"	J	0	0	0	0	0	0
"	T	0	0	0	0	0	0
Serum H unabsorbed	H	4	4	4	4	4	3
" " "	R	4	4	4	4	4	3
" " "	L	4	4	4	4	3	3
" " "	E	4	4	4	4	4	2
" " "	Ma	4	4	4	4	3	2
" " "	Mc	4	4	4	4	4	4
" " "	J	0	0	0	0	0	0
" " "	T	0	0	0	0	0	0
Serum H absorbed by Strain H	H	0	0	0	0	0	0
" " " " " R	R	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " L	L	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " E	E	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " Ma	Ma	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " Mc	Mc	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " J	J	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " T	T	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0

homologous agglutinin could not be completely absorbed by these other strains. On the other hand, when Serum H produced by the strain from another nurse was similarly tested, it was found that all of the patients' strains which were in the preliminary agglutinative group completely absorbed the agglutinin, from this serum. Likewise, when the sera produced by the patients' strains were absorbed by the six different strains from the noses or throats of the six nurses, Strain H, from one nurse's nose, was the only one of the six which would completely absorb the agglutinin from these sera. Of the eight patients' strains used in the last series, five, namely R, L, E, Ma and Mc, produced sera which, with Serum H from the nurse's nose Strain H, gave complete cross agglutination and cross absorption of agglutinin tests. The absorption tests of these six strains with the serum produced by the nurse's strain is shown in Table II. Similar results were obtained with the other five sera produced by the five patients' strains mentioned above. Thus the criteria for the demonstration of the antigenic identity of these six strains were established.

The sera from the two blood culture Strains J and T persistently failed to agglutinate the homologous strains but did agglutinate fully the six identical strains. Furthermore, the two strains J and T producing these sera completely absorbed the agglutinin out of the six identical sera. This will be discussed below. One patient's strain produced a serum which agglutinated the other strains irregularly and this strain did not absorb the agglutinin from the six identical sera. The other two nurses' strains which were included in the preliminary agglutinating group were slow to produce high titred serum and when it was found that these failed to absorb the agglutinin from the six identical sera, they were discarded.

When agglutination and absorption of agglutinin tests were done with the other strains which had fallen into the main agglutinative group in the preliminary tests but against which no immune sera had been prepared, it was found that sixteen more strains completely agglutinated in Serum H produced by the nurses' nose strain and completely absorbed agglutinin in the same absorptive dose as the homologous strain. These are shown in Table III. Six of these strains had come from other foci in four of the patients whose original culture had already been demonstrated to be antigenically identical

with Strain H. Nine of these sixteen strains had come from eight other patients, two of whom had positive vaginal cultures without clinical signs of puerperal fever. One of the sixteen strains came from the axillary abscess of the nurse who had pricked her finger with a pin.

TABLE III.

Final Classification of 54 Strains of Hemolytic Streptococci.

Antigenically identical		Probably identical		Not classifiable	
Complete reciprocal agglutination and agglutinin absorption tests		Complete absorption of agglutinin		No absorption of agglutinin nor agglutination	
Strain	Source	Strain	Source	Strain	Source
L1	Patient's blood	L2	Patient's vagina	Wh	Patient's vagina
Mc1	" "	L3	" peritoneum	Ce	" "
Ma1	" vagina	Mc2	" vagina	Co	" "
R	" "	Mc3	" pleura	Se	" "
E1	" "	Ma2	" abscess	Sc	" "
H	Nurse's nose	E2	" "		And 22 other strains from nose and throat hospital staff
		J1	" blood		
		J2	" vagina		
		T1	" blood		
		T2	" vagina		
		D	" "		
		Ba	" "		
		Wo	" "		
		F	" "		
		P	" "		
		N	" "		
		Bl	" "		
		O'M1	" "		
		O'M2	" thigh		
		M	Nurse's abscess		
		Sm	" peritoneum		

Aside from the sixteen strains, which both by the agglutination and by the agglutinin absorptive tests demonstrated their close antigenic relationship with Strain H, five strains completely absorbed the agglutinin from Serum H, but persistently failed to agglutinate in it. Two of these, Strains J and T, as stated above, came from the blood cultures of two of the fatal cases and two more came from the vaginæ of the same two patients. The fifth came from the peritoneum of

the nurse who developed hemolytic streptococcus peritonitis during the course of the epidemic. In the preliminary tests this strain had agglutinated in the sera produced by the patients' strains.

Five other patients' strains failed either to agglutinate or to absorb agglutinin although one of these, Strain Wh, in the preliminary tests had agglutinated in all of the sera produced by the patients' strains. When this last strain was again tested against these sera it failed both to agglutinate and to absorb agglutinin from them. The results with this strain were irregular both for agglutination and agglutinin absorption. Likewise the serum produced by it gave irregular results. It may have been an incidental strain or have degenerated during the progress of the tests.

DISCUSSION.

It is of interest to note the sources from which the organisms were obtained. Even for those strains which were demonstrated to have the closest antigenic relationships it is evident that the organisms had no specific affinity for any particular organ or tissue and could reside for a time at least in the nasal passage as well as in the vaginal passage without producing clinical symptoms. On the other hand it is evident that the organisms producing the epidemic were in a state of high virulence or invasiveness, for the mortality of those afflicted with the disease was high. Likewise the onset of symptoms was rapid. This was true in the case of the nurse who pricked her finger with a pin and in whom we probably know the exact time of the introduction of the organisms (20 hours between the prick of the finger and the onset of symptoms) as well as in the case of the patients with puerperal fever and the nurse with peritonitis in whom we do not know the exact time of introduction. In rabbits also the organisms proved to be highly invasive (although there would be no reason to expect a parallel susceptibility) for even after a month or more of vaccination, several animals developed septicemia and died following the first small injection of living organisms. This fact would seem to argue against the rationale of specific vaccination during the acute stages or even the chronic stages of the disease itself, as advocated by some. The normal animals could not build up protective substances rapidly against these organisms following intravenous

injection. Whether or not they would have done so, had we injected the organisms by any other route, we cannot tell.

Considering first the results of the preliminary agglutination tests we find that twenty-one out of thirty-one strains cultured from patients, were included in the main agglutinating group. Our chief concern here is to explain the failures if that be possible. Of the ten which failed to agglutinate, one came from the nose and one from the throat of two parturient women without post partum fever after the epidemic was over, being discovered by routine cultures of the nose and throat of all cases admitted for delivery. While it is possible that these patients picked up these organisms after admission into the hospital, it is more likely that they brought them in when they entered and they are evidently not related to the epidemic. Four of the ten strains came from the vaginæ of patients with puerperal fever who were frankly considered to be included in the epidemic. It is possible that these cases were caused by organisms different from the strains responsible for the epidemic and were simply coincident with but not a part of the epidemic. On the other hand, in view of the changes which we know took place in the antigenic and agglutinative characteristics of some of the other strains it is probable that some of these at any rate, failed to agglutinate because they had undergone certain degenerative processes in the course of several months habitation in artificial media. Krumwiede has brought out the fact that these changes do occur with other organisms and it has been observed by others. In such circumstances it is readily understood that although positive results with agglutination and absorption of agglutinin tests are significant of similarity of antigenic properties, negative results do not necessarily prove that similarity did not exist at the time of the first cultivation. It would seem advisable to emphasize this point by repetition namely that positive results mean something but negative results mean nothing, for although strains once positive may become negative, strains originally negative and antigenically different if they change at all, have an infinitely small chance of becoming positive and antigenically identical with another strain.

The other four strains of the ten failures which we are now considering, came from two of the fatal cases (J and T) in the early part of the epidemic, two were vaginal cultures and two were blood cul-

tures. One of these patients (T) was especially cared for by the nurse who pricked her finger with a pin. We have reason to believe that these strains originally were the same as the ones which we have definitely proved to be antigenically identical. The following facts with regard to the behavior of these strains lend weight to this belief. One of these strains, namely J from a blood culture, was used to produce one of the preliminary sera. During the course of inoculation, the serum of the rabbit was tested from time to time and showed gradually increasing agglutinative titre for the homologous strain. When it had attained the desired titre, mentioned above, the animal was killed by bleeding and the serum was taken and stored. About a month's time elapsed before this serum was tested for agglutination with all of the strains and then to our surprise the serum failed to agglutinate the homologous Strain J but ran parallel with the other sera produced by the other patients' strains in that it agglutinated the same strains. Evidently something had happened to Strain J in the meanwhile. On thinking back it was recalled that some difficulty had been encountered in recovering the organism out of the stock culture. It had evidently died off in the supernatant fluid of the cooked meat medium and could only be recovered by transplanting some of the meat. After this, it was noted that the organism was not as clearly hemolytic and grew more diffusely than before. The same thing occurred with several of the strains, notably all four of the strains we are now considering, also the strain from the peritoneum of one nurse and the strain from the axillary abscess of the other nurse as well as two of the four strains from non-fatal cases which, as mentioned above, failed to fall into the main group. Whether this feature of their cultivation, namely their more rapid death in the meat medium than other strains, depended upon some slight variation in the media or in some inherent quality in the strain itself, cannot be determined but the fact that both the blood and the vaginal cultures from two patients should do the same thing, would suggest that the latter explanation is at least a possible one. It is even possible that such changes may have taken place while the organism multiplied within the patient. This cannot be the sole factor however because Strain J did agglutinate in the homologous serum at first and the change came later.

Both of these blood culture Strains J and T, as has been stated above, were included in the group of strains selected for the second series of agglutinative sera. During the course of inoculation these strains were tested for agglutination with their corresponding sera and persistently refused to agglutinate. After continuing the vaccinations for more for 2 months, the sera were tested for agglutination with the six other strains which had by that time demonstrated their antigenic identity. To our surprise, both of these sera fully agglutinated all of these six strains and when these two blood culture Strains J and T were used for absorption, it was found that they both completely absorbed the agglutinin out of the sera produced by the other strains. This is shown in Table II (*q. v.*). We have, therefore, two strains which are antigenic in the sense that they can produce agglutinating serum. They can also combine with the antibody in serum and carry it down during centrifuging but they persistently refuse to agglutinate. They take part in the first stage of the agglutination process by uniting with the antigen but the second stage, which is the visible one of flocculation and sedimentation, does not take place. We can only be certain of the fact of their union with the antibody by testing the absorbed serum and finding that the agglutinin antibody has been removed. We have, then, two sera for blood culture Strain J, one produced while the organism was agglutinable and one produced after it had lost its agglutinability. So far no differences in agglutinin action have been found in these two sera. The split in the two phases of agglutination as demonstrated by these results when combined with other facts, may throw some light upon the phenomenon as a whole and upon the antigenic characteristics of hemolytic streptococci.

If we turn to Table I to consider the results of the preliminary agglutination tests with the strains cultured from the staff, we find that nine of these were included in the main agglutinative group. Six of these failed to absorb the agglutinin completely from any serum but their own. One (Strain H) from the nose of a nurse was found, by the reciprocal agglutination and absorption of agglutination tests, to be antigenically identical with five of the patients' strains. One (Strain MO) from the axillary abscess of the nurse who pricked her finger, in the later tests lost some of its agglutinability but com-

pletely absorbed agglutinin. The last (Strain S) from the peritoneum of the nurse with so called primary peritonitis completely lost its agglutinability in the later tests and yet completely absorbed agglutinin from the serum produced by Strain H, one of the six strains proved antigenically identical. No serum was produced for these last two strains so that we are not able to carry out reciprocal tests but the fact of their power to completely absorb agglutinin makes probable their antigenic identity with the other strains.

When the tests had been completed the question obviously arose whether the nurse, whose nose culture yielded Strain H, which proved to be antigenically identical with five of the patients' strains, was or was not the one who brought the organism into the hospital. It was found on examining her record that she had not joined the staff until after the epidemic had started. Whether or not she aided in its continuance cannot be stated. The fact is demonstrated, however, that the organism could be carried in the nose of a staff member without producing clinical symptoms.

Of the fourteen strains cultured from the staff which failed to fall into the preliminary group, certain ones may have originally been in the epidemic group and have lost their antigenic characteristics between the time of the isolation and the tests. The changes which we know took place in some of the other strains make it not at all unlikely that this is so. The original carrier may have been in this group but this can never be determined.

SUMMARY.

1. A study has been made by means of agglutination and absorption of agglutinin tests, of the antigenic relationships of 54 strains of hemolytic streptococci associated with an epidemic of puerperal fever in a large city obstetric hospital.

2. These organisms were cultured from the blood, vaginæ, peritoneal cavities and metastatic foci of patients, from the noses and throats of patients and staff members and from the peritoneum and axillary abscess of two of the nurses.

3. In preliminary agglutination tests twenty-one out of thirty-one cultures from the patients and nine out of twenty-three cultures from the hospital personnel, fell into a single agglutinative group.

4. By reciprocal agglutination and absorption of agglutinin tests, six of these strains were demonstrated to be antigenically identical. Two of these came from patients' blood cultures, three from patients' vaginal cultures and one from the nasal culture of one of the nurses. This nurse was, however, not the original carrier for she did not join the hospital staff until after the epidemic began.

5. By agglutination and complete absorption of agglutinin, sixteen more strains showed their antigenic similarity to, if not identity with, these other six strains. Four of these sixteen came from metastatic foci and two from the vaginæ of four of the five patients which had yielded from another source, the identical strains mentioned in 4. Nine came from eight other patients and one from the axillary abscess of a nurse. No reciprocal tests were possible with these sixteen strains because sera had not been prepared against them.

6. Five other strains which completely absorbed agglutinin had evidently lost their ability to agglutinate. Two of these strains were from the blood cultures and two from the vaginal cultures of two fatal cases. One was from the peritoneal cavity of a nurse.

7. Two of these five strains when injected into rabbits in the usual way for the purpose of producing agglutinating antiserum, stimulated the production of sera which agglutinated completely the six antigenically identical strains but failed to agglutinate the homologous strains used to produce the respective sera.

8. These two strains therefore retained the antigenic properties first of stimulating the agglutinating antibody in normal animals and second of uniting with the agglutinating antibody in the serum but lost the ability to bring about the second phase of agglutination —namely flocculation.

9. We have demonstrated, therefore, a more or less permanent splitting of the phenomenon of agglutination.

CONCLUSIONS.

1. A series of twenty-four cases of puerperal fever occurring in the course of 1 month in a certain obstetric hospital constituted a small epidemic apparently caused by a single strain of hemolytic streptococcus of unusual virulence.

2. An organism capable of producing puerperal fever may be carried in the nose of one of the hospital staff without causing clinical symptoms of disease.

3. Careful masking of both nose and mouth should be practiced by all those attendants who may come in contact with parturient women either just before, during or just after labor.

4. Strains of hemolytic streptococci may lose some of their antigenic and agglutinative characteristics if kept on certain artificial media for a period of some months.

5. They may retain their antigenic power both to stimulate agglutinin and combine with agglutinin and at the same time lose their ability to agglutinate.

6. Biological studies employing the agglutination or absorption of agglutinin tests should be carried out as soon as possible after the isolation of the organisms.

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